A cytosolic factor mediating membrane recruitment of AP-1 clathrin adaptors

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Summary

Transport of cargo within the endocytic and secretory pathway is generally mediated by coated vesicles. These vesicles are formed through the recruitment of cytosolic coat proteins to the donor membrane that act as a scaffold to form coated buds and vesicles. At the same time they selectively concentrate cargo proteins by interacting with cytosolic signals. Clathrin, in combination with different adaptor proteins (APs), is the major coat protein for vesicle formation at the plasma membrane, endosomes and the trans-Golgi network. Best characterized is clathrin mediated endocytosis at the plasma membrane which involves AP-2 and a network of associated proteins. Much less is known about AP-1 mediated clathrin coated vesicle formation at the TGN/endosomes.

In vitro studies demonstrated that the minimal requirements to recruit AP-1 to liposome membranes are activated Arf1, phosphoinositides, and either sorting signals or an unknown cytosolic factor. In order to identify this factor, we fractionated calf brain cytosol by several chromatographic methods. Fractions were tested for factor dependent AP-1 recruitment activity using an in vitro assay. Purification via ammonium sulfate precipitation, hydrophobic interaction chromatography, anion/cation exchange chromatography or hydroxyapatite chromatography produced a final fraction containing three major proteins: amphiphysin 1, amphiphysin 2 and endophilin A1. All three proteins are known accessory factors in clathrin coated vesicle formation at the plasma membrane. Co-immunodepletion of amphiphysin 1 and 2 resulted in a strong reduction of AP-1 recruitment activity. Therefore we conclude that a heterodimer of amphiphysin 1 and 2 is the long searched for cytosolic factor, required to recruit AP-1 in the absence of sorting signals in vitro. Our results strongly suggest that amphiphysin 1, amphiphysin 2 and endophilin A1 are also involved in AP-1 mediated clathrin coated vesicle formation at the TGN and endosomes in vivo.

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Abbreviations

α-SNAP	Soluble NSF attachment protein α
γ-BAR	γ1-adaptin brefeldin A resistance
AAK1	Adaptor-associated kinase 1
AMPH1	Amphiphysin 1
AMPH2	Amphiphysin 2
ANTH	AP180 N-terminal homology domain
AP-1, -2, -3, -4	Adaptor protein 1, 2, 3, 4
ARF	ADP-ribosylation factor
ARFGAP1	ADP-ribosylation factor GTPase-activating protein 1
ARH	Autosomal recessive hypercholesterolemia
Arp 2/3	Actin-related proteins 2/3
BAR	Bin1, Amphiphysin, and Rvs167
BFA	Brefeldin A
BSA	Bovine serum albumin
BiP	Binding protein
CALM	Clathrin assembly lymphoid myeloid leukaemia protein
CD	Cation-dependent
Chs3p	Chitin synthase 3
Chs5p	Chitin synthase 5
Chs6p	Chitin synthase 6
ChAPs	Chs5p-Arf1p-binding proteins
CHC	Clathrin heavy chain
CI	Cation-independent
CK2	Casein kinase II
CLASPs	Clathrin-associated sorting proteins
CLAP	Clathrin/AP-2-binding region
CLIC	Clathrin and dynamin independent carriers
COPI / II	Coat protein I / II
BSA	Bovine serum albumin
CCV	Clathrin coated vesicle
CK2	Casein kinase II
Dab2	Disabled-2

DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDEM	ER degradation-enhancing 1, 2-mannosidase-like protein
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
EEA1	Early endosome antigen 1
EGF(R)	Epidermal growth factor (receptor)
EH	Eps15 homology
EM	Electron microscopy
ENTH	Epsin N-terminal homology
EPS15	Epidermal growth factor protein substrate 15
Epsin1	EPS15 interacting protein
EpsinR	Epsin related protein
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERES	ER exit sites
ERGIC	ER-Golgi intermediate compartment
ESCRT	Endosomal sorting complex required for transport
F-actin	Filamentous actin
FAPP1/2	Phosphatidylinositol-four-phosphate adaptor protein-1/2
FPLC	Fast protein liquid chromatography
FT	Flow through
GAGs	Glycosaminoglycans
GAK	Cycling G-associated kinase
GAP	GTPase activating protein
GAT	GGA and Tom
GBF1	Golgi-specific brefeldin A resistance factor 1
GDI	GDP dissociation inhibitor
GDF	GDI displacement factor
GEF	Guanine nucleotide exchange factor
GGA	Golgi-localized, γ ear-containing, Arf binding protein
GMP-PNP	Guanylyl imidodiphosphate
GPCR	G-protein coupled receptor
GPI	Glycophosphatidylinositol

GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
GTP	Guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HIC	Hydrophobic interaction chromatography
Hsc70	Heat shock cognate protein 70
IF	Immunofluorescence
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranosid
Lamp-1	Lysosome-associated membrane protein 1
LIMPII	Lysosomal integral membrane protein II
LDL(R)	Low density lipoprotein (receptor)
MW	Molecular weight
M6P	Mannose 6-phosphate
MES	2-(*N*-morpholino)ethanesulfonic acid
MPR	Mannose 6-phosphate receptor
MBVs	Multivesicular bodies
MMCC-DOPE	(N-((4-maleimidylmethyl)cyclohexane-1-carbonyl)-1,2-dioleolyl-sn-glycero-3-phosphoethanolamine
NBD-PE	(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine
NSF	N-ethylmaleimide-sensitive-factor
N-WASP	Neural Wiskott-Aldrich syndrome protein
NTID	N-terminal insert domain
PA	Phosphatidic acid
PACS-1	Phosphofurin acidic cluster-sorting protein
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PDI	Protein disulfide isomerase
PE	Phosphatidylethanolamine
PH	Pleckstrin homology domain
PI	Phosphatidylinositol
PIC	Protease inhibitor cocktail
PIP	Phosphoinositide
PM	Plasma membrane

PMSF	Phenylmethylsulfonylfluorid
PP2A	Protein phosphatase 2A
PRD	Proline-rich domain
PS	Phosphatidylserine
PtdIns(3)P	Phosphatidylinositol (3)-monophosphate
PtdIns(4)P	Phosphatidylinositol (4)-monophosphate
PtdIns(4,5)P_2	Phosphatidylinositol (4,5)-bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol (3,5)-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5)-trisphosphate
PX	Phox homology domain
ROCK2	Rho associated protein kinase 2
Sar1	Secretion-associated and Ras-related protein 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3	Src homology 3 domain
SNAP	Soluble NSF attachment protein
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNX	Sorting nexin
SRP	Signal recognition particle
TfR	Transferrin receptor
TGN	Trans Golgi network
VHS	Vps, Hrs, and STAM
VSV-G	Vesicular stomatitis virus G protein

Introduction

1. Intracellular transport

Eukaryotic cells use an elaborate internal membrane system to transport proteins and lipids along the secretory and endocytic pathway (Figure 1). This intracellular transport is very efficient and highly specific. The identity of organelles is defined by their protein and lipid composition. Therefore, it has to be ensured that cargo is delivered in a highly selective manner to the appropriate compartment.

Although a lot of the molecular details of the intracellular transport machinery are still not well understood, considerable progress has been made over the last decades towards understanding the molecular basis of membrane traffic in the secretory and endocytic pathway.

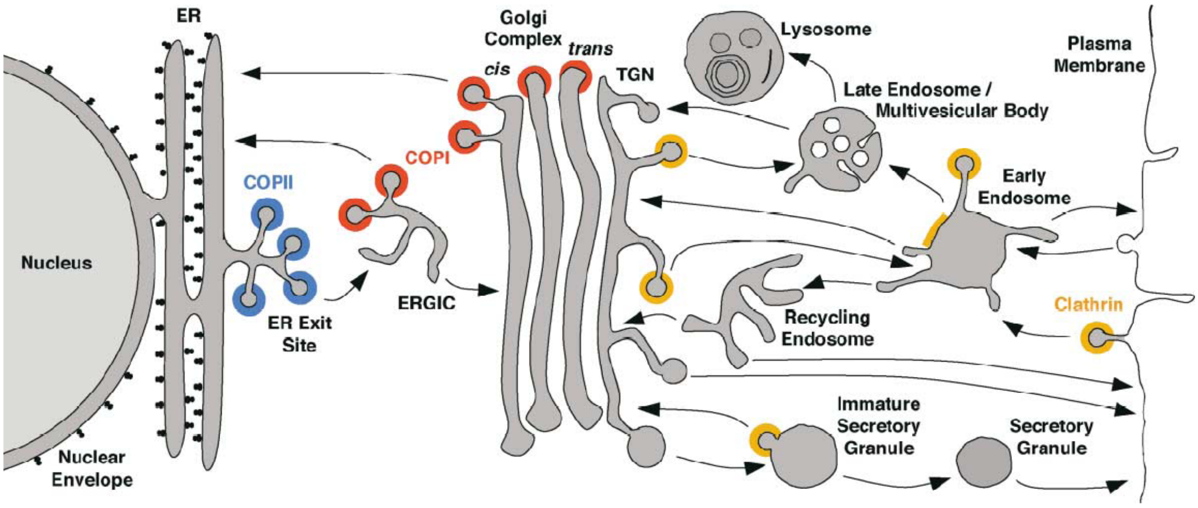

Figure 1. **Intracellular transport pathways in eukaryotic cells:**
The scheme depicts the internal membrane system of a eukaryotic cell that allows the transport of cargo along the secretory and endocytic pathways. The distinct compartments are interconnected through different transport steps (indicated by arrows). The colours indicate the known or presumed location of the membrane associated coat proteins COPII (blue), COPI (red), and clathrin (yellow). Additional coats or coat like structures also exist, but are not represented in this figure. (Reproduced from Bonifacino and Glick, 2004).

1.1 The secretory pathway

The secretory pathway of eukaryotic cells consists of several membrane enclosed compartments which regulate the delivery of newly synthesized proteins, carbohydrates and lipids to the cell surface – a necessity for growth and homeostasis. Secretory cargo is synthesized and assembled at the endoplasmatic reticulum (ER) from where it is transported through the ER to Golgi intermediate compartment (ERGIC) to the Golgi apparatus where it is further processed. Finally it arrives at the TGN, where cargo is sorted into post-Golgi carriers that ultimately fuse with the plasma membrane (Bonifacino and Glick, 2004). Since in this anterograde transport pathway membranes and also some organelle specific proteins are constantly removed from their compartment of origin, a retrograde pathway has to counteract this process in a highly specific manner.

The secretory pathway machinery can be subdivided into four distinct steps: 1. ER import and quality control; 2. ER to Golgi transport; 3. intra Golgi transport/ER retrieval and 4. post-Golgi transport.

1.1.1 ER import and quality control

The ER is the largest organelle of the cell. It consists of an extensive array of interconnecting membrane tubules and cisternae that extend throughout the cell, including the nuclear envelope, and can be subdivided into rough and smooth regions, depending on whether ribosomes are associated with their cytoplasmic surfaces. The functions of the ER include protein folding, assembly and degradation, lipid metabolism, detoxification and calcium regulation.

Proteins destined for the secretory pathway are cotranslationally translocated. They contain a hydrophobic signal sequence of 7-25 amino acids typically located at the N-terminus, which is recognized by the signal recognition particle (SRP) as soon as it has emerged from the ribosome. This transiently arrests elongation and targets the ribosome-nascent polypeptide chain-SRP complex to the ER membrane via interaction with a transmembrane SRP receptor (Gilmore et al., 1982; Meyer and Dobberstein, 1980; Walter et al., 1982). Binding to this receptor targets the ribosome-nascent polypeptide chain complex to the Sec61 translocation complex which forms an aqueous protein conducting channel. SRP and its receptor are released and the translation of the polypeptide continues through the translocon pore into the ER lumen.

The ER lumen provides the optimal environment for protein folding and modification. Cotranslational and posttranslational modifications include disulphide bond formation, N-linked glycosylation and glycophosphatidylinositol (GPI)-anchor addition. To ensure that only correctly folded proteins leave the ER, there exists a strict quality control system. Molecular chaperones that are used in quality control include the immunoglobulin binding protein (BiP), calnexin/calreticulin and protein disulfide isomerase (PDI). They bind unfolded proteins and keep them in the ER. At the same time they facilitate folding reactions necessary for protein maturation and oligomerization so that correctly folded proteins can finally be released (Helenius et al., 1992). Calnexin and calreticulin for example are lectins that bind monoglucosylated, trimmed intermediates of the N-linked core glycans on newly synthesized glycoproteins (Figure 2). The thiol-disulphide oxidoreductase ERp57 associates with the two chaperones and catalyzes disulphide-bond formation on their substrate. Upon removal of the last glucose by Glucosidase II the chaperone complex dissociates from the glycoprotein. If the protein is still not correctly folded, it is reglucosylated by UDP-glucose:glycoprotein glucosyltransferase so that the chaperones can reassociate with it. Thus an unfolded glycoprotein undergoes continuous cycles of glucosylation and deglucosylation until it has either reached its native conformation or is targeted to ER-associated degradation (ERAD). This requires the trimming of a single mannose in the middle branch of the oligosaccharide which leads to an association with ER degradation-enhancing 1, 2-mannosidase-like protein (EDEM) and retrotranslocation into the cytosol where the protein is rapidly ubiquitylated before it is digested via proteasomes.

In contrast, correctly folded proteins destined for the secretory pathway are actively sorted into ER exit sites and taken up by COPII vesicles, the first class of coated vesicles involved in secretory transport (Barlowe et al., 1994).

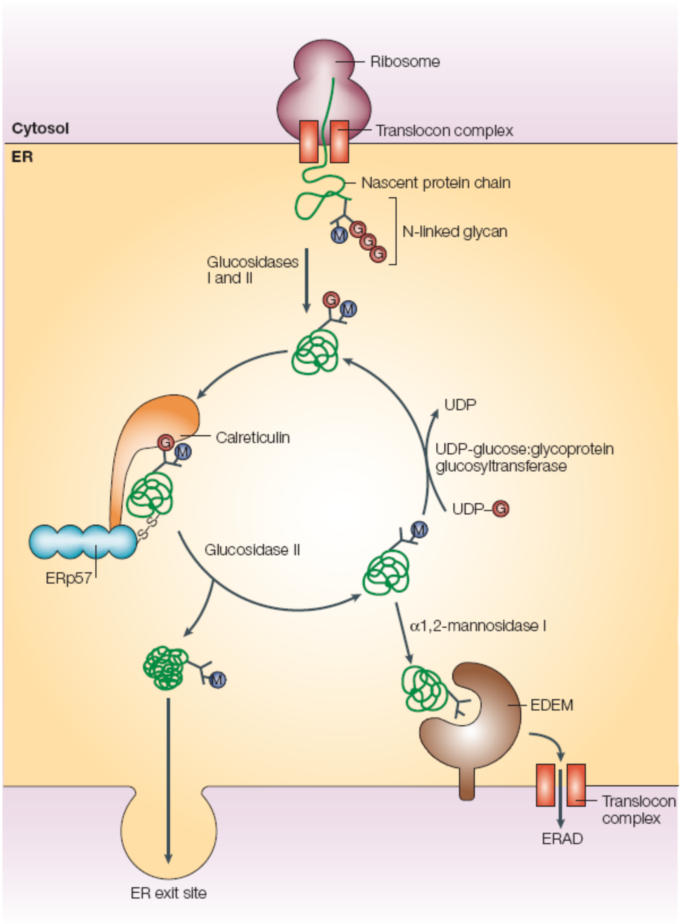

Figure 2. **The calnexin/calreticulin cycle.**

Calnexin and calreticulin assist in the proper folding of glycoproteins in the ER. For simplicity, only calreticulin is depicted. The circles represent glucose (red circles) and mannose (blue circles) residues on the different intermediates of the N-linked core glycans. (Reproduced from Ellgaard and Helenius, 2003).

1.1.2 ER to Golgi transport

COPII vesicles transport secretory cargo from the ER to the ERGIC, which is defined by the presence of the lectin Ergic53 (Hauri et al., 2000). The ERGIC consists of a few hundred tubulovesicular membrane clusters in the vicinity of ER exit sites (Appenzeller-Herzog and Hauri, 2006). It is still unclear whether the ERGIC forms de novo by homotypic fusion of COPII vesicles or if it is a pre-existing compartment which takes up newly made COPII vesicles via heterotypic fusion (Bannykh and Balch, 1998). It is believed that the ERGIC represents the first entity that discriminates between anterograde and retrograde transport, since cargo destined for retrieval to the ER is concentrated in COPI positive structures, whereas secretory cargo is predominantly found in COPI negative regions (Martinez-Menarguez et al., 1999). Anterograde transport from ERGIC to the cis-Golgi was shown to be mediated by pleiomorphic vesicles (Ben-Tekaya et al., 2005). However, the mechanism of the formation of these vesicles remains unknown.

1.1.3 Intra Golgi transport/ER retrieval

The Golgi apparatus is composed of 4-6 membrane enclosed flat cisternae that are grouped into several stacks linked by tubular connections between corresponding cisternae. In mammalian cells the Golgi complex is normally localized near the cell nucleus and close to the centrosome due to interactions with microtubules. It can be subdivided in cis-, medial and trans-Golgi. Proteins enter the Golgi from the cis-side. During their transport through the different cisternae to the trans-side, Golgi resident proteins sequentially modify N-linked carbohydrate chains and add O-linked oligosaccharides. Furthermore glycosaminoglycans (GAGs) are synthesized and attached to proteins to produce proteoglycans that are finally delivered to the extracellular matrix. The Golgi is also important for labelling proteins destined for lysosomes with a mannose 6-phosphate, a signal that is later recognized by the mannose 6-phosphate receptors. On the trans-side, the Golgi is connected with the trans-Golgi network (TGN), where secretory proteins are sorted to their final destination.

Two alternative models for intra Golgi transport have been proposed. In the cisternal maturation model, anterograde cargo is transported en bloc with cisternae (Glick and Malhotra, 1998; Matsuura-Tokita et al., 2006). New cisternae would assemble at the cis-Golgi and then mature along the Golgi apparatus until they would disassemble at the trans-Golgi. COPI vesicles would transport resident enzymes from the more trans to the more cis located cisternae.

In the vesicular transport model, in contrast, the cisternae are static and anterograde as well as retrograde transport is mediated via specific COPI vesicles (Rothman and Wieland, 1996).

Most probably transport occurs by a combination of the 2 models, probably with a slow anterograde transport mediated by maturation and a fast anterograde transport mediated by vesicular transport (Orci et al., 2000; Pelham and Rothman, 2000).

In the Golgi, proteins and lipids destined for the ER/Golgi system have to be separated from those to be delivered to the plasma membrane or the endosomal/lysosomal system. ER resident membrane proteins contain cytosolic signals that are recognized by COPI and are thus packaged into COPI coated vesicles for retrograde transport to the ER. Soluble ER resident proteins, such as BiP in contrast contain a KDEL signal sequence at their C-terminus which is recognized by the KDEL receptor (Munro and Pelham, 1987) and subsequently sorted into COPI vesicles (Majoul et al., 1998; Pelham, 1988).

1.1.4 Post Golgi transport

The trans-Golgi network (TGN) is a tubular network that originates from the last two trans-Golgi cisternae and makes close contact with the ER, most probably for lipid exchange (Ladinsky et al., 1999; Mogelsvang et al., 2004). At the TGN, secretory proteins receive their final posttranslational modifications (e.g. sulfation), before they are sorted into distinct pleiomorphic carriers that are targeted to different destinations (Griffiths and Simons, 1986; Rodriguez-Boulan and Musch, 2005). At the same time the TGN receives cargo from endosomes and the plasma membrane (Bonifacino and Rojas, 2006). Thus, the TGN combines secretory and endocytic routes.

There exist different exit routes for secretory cargo at the TGN (Figure 1). In the constitutive pathway, which exists in all cell types, cargo is transported to the apical or basolateral plasma membrane, early endosomes and late endosomes. Endocrine and neuroendocrine cells contain an additional regulated secretory pathway where cargo is sorted into secretory granules that fuse with the plasma membrane upon an external stimulus (reviewed in Tooze et al., 2001). To ensure correct cargo transport, secretory proteins are sorted by different mechanisms like sorting signals, posttranslational modifications, aggregation or by the affinity for specific membrane domains, and segregated into different TGN domains. After cargo segregation, cargo-containing tubular domains are generated by protein and lipid based mechanisms that may include the curvature inducing proteins of the BAR family. Such TGN export domains finally interact with suitable microtubule based motors (usually kinesins) and are drawn out from the Golgi along microtubules, followed by fission of the tubular precursor into centrifugally moving carriers. Fission can take place anywhere along the tubule which explains the pleiomorphic and variable aspects of these carriers (De Matteis and Luini, 2008). In a similar way, endosome directed carriers can detach from the TGN as simple vesicles or as entire pieces of TGN membranes containing 2-3 clathrin coated buds.

1.2 The endocytic pathway

Endocytosis is a basic cellular process that has an essential role in delivering membrane components, receptor associated ligands and soluble molecules to various intracellular destinations. There are several pathways for internalizing cargo from the cell surface. Some of them are constitutive, whereas others are triggered by specific signals. The best studied endocytic process is the clathrin-dependent internalization of receptors and their ligands. Other, clathrin-independent internalization routes include actin based macropinocytosis and phagocytosis, and caveolin dependent endocytosis depicted in Figure 3.

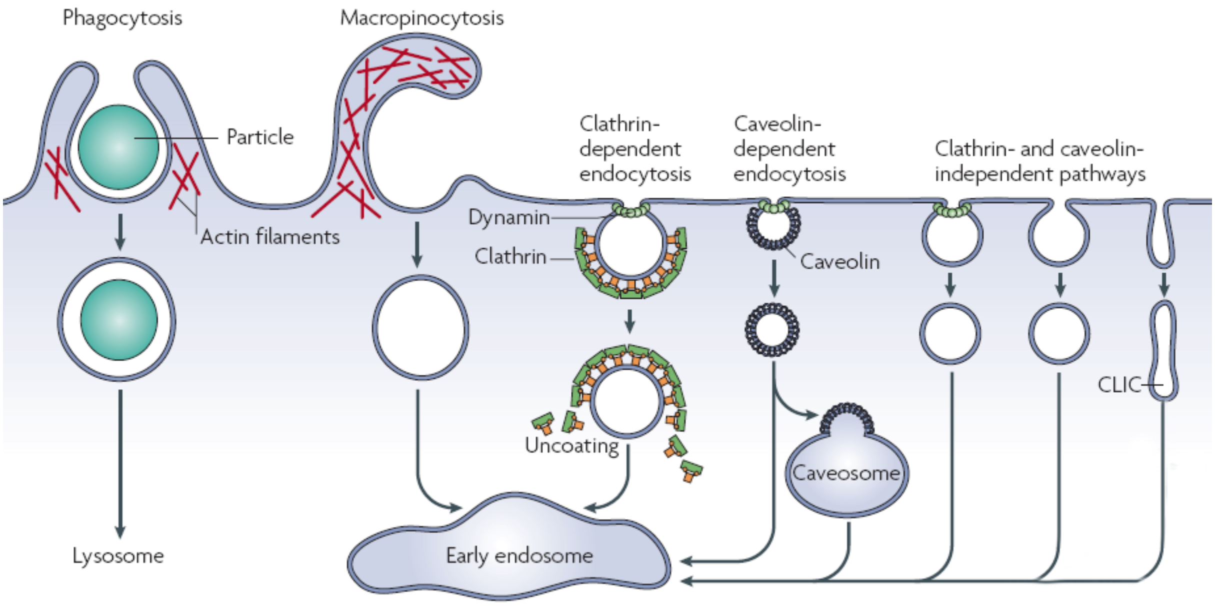

Figure 3. **A model of the different endocytic pathways in a cell:**
Large particle and fluid uptake occurs via phagocytosis and macropinocytosis, respectively. Other cargo is taken up by different endocytic mechanisms like clathrin and caveolin dependent endocytosis. However, numerous cargo can also be endocytosed by mechanisms that are independent of clathrin and caveolin. Most internalized cargo is delivered to the early endosome via vesicles or tubular intermediates (clathrin and dynamin independent carriers (CLICs)). This occurs either directly from the plasma membrane or indirectly over intermediate compartments like the caveosome. (Adapted from Mayor and Pagano, 2007).

Most internalized cargo is delivered to early endosomes via vesicular or tubular intermediates (known as clathrin and dynamin independent carriers (CLIC)) (Mayor and Pagano, 2007). Early endosomes can be subdivided into peripherally localized sorting endosomes and recycling endosomes. The former represent the first main branch point in receptor mediated endocytosis. Cargo receptors that enter the sorting endosome release their ligands due to the lower pH and are then either recycled back to the plasma membrane directly from the sorting endosome (fast pathway) or indirectly over recycling endosomes (slow pathway). Ligands, other solutes and receptors that are not recycled stay in the sorting endosome, which stops the fusion with newly endocytosed material and translocates along microtubules to the centre of the cell. Simultaneously, it becomes more acidic and matures into the late endosome (Maxfield and McGraw, 2004). Along the pathway to the lysosome, ESCRT (endosomal sorting complex required for transport) protein complexes I-III function sequentially in sorting proteins into intralumenal vesicles. Thus, the late endosome containing these intralumenal vesicles is referred to as multivesicular bodies (MVBs) (reviewed in Saksena et al., 2007). Fusion of the MVBs with the lysosome finally delivers these vesicles and their content into the lumen of the lysosomes where the vesicles and cargo are degraded.

Endosomes also play an important role in retrograde transport to the TGN. Acid-hydrolase receptors (e.g. mannose 6-phosphate receptors), transmembrane enzymes (e.g. furin) and SNARES (e.g. VAMP4) are known substrates for retrograde transport from late and recycling endosomes to the TGN (Saksena et al., 2007).

2. Coated transport vesicles

The best studied transport intermediates that transfer cargo from a specific donor organelle to the appropriate target membrane are COPI-, COPII-, and clathrin-coated vesicles (CCVs).

These small spherical carriers are classified according to their protein coat, and mediate transport in different intracellular pathways. The main function of all three coat proteins is the same. On the one hand they participate in cargo selection by recognizing cytosolic sorting signals in transmembrane cargo. On the other hand they help to deform the flat membrane into round buds to finally release a coated vesicle. After budding, the coat disassembles to allow the transport vesicle to fuse with its target membrane and release its cargo.

2.1 COP I vesicles

COPI coated vesicles are primarily involved in the retrograde transport of cargo between the Golgi and ER (Cosson and Letourneur, 1994; Letourneur et al., 1994; Majoul et al., 2001). They also mediate transport within the cisternae of the Golgi apparatus, however, the exact role of COPI vesicles within intra Golgi transport is still not exactly clear.

The COPI coatomer consists of seven cytosolic subunits (α, β, β', γ, δ, ε and ζ) which can be separated into two functionally different subcomplexes, the F-COPI (β, γ, δ, ζ) and the B COPI (α, β', ε) (Fiedler et al., 1996). The four subunits of the F-COPI seem to be related in sequence and structure to the clathrin binding AP complexes (Boehm and Bonifacino, 2001; Eugster et al., 2000; Hoffman et al., 2003; Watson et al., 2004) whereas the B-COPI subunits seem to function as a structural scaffold like clathrin (see below). There exist at least three different coatomer complexes involving two different isoforms of γ COP and ζ COP (γ2 and ζ2) (Blagitko et al., 1999; Futatsumori et al., 2000). It is thought that these different coatomer isotypes increase the cargo repertoire of COPI vesicles through interactions with different sorting signals.

The initial step in COPI vesicle biogenesis is the recruitment and activation of the cytosolic GTPase Arf1. It is thought that the transmembrane protein p23 and probably p24, both members of the p24 family, may act as primary Arf1-GDP receptors (Contreras et al., 2004; Gommel et al., 2001; Majoul et al., 2001). Additionally the specific association of Arf1 with its appropriate guanine nucleotide exchange factor (GEF) ensures targeting to the correct membrane. Several GEFs for Arf1 have been identified, with GBF1 being probably the relevant GEF involved in COPI biogenesis at the cis-Golgi (Niu et al., 2005; Zhao et al., 2002). GBF1 stimulates Arf1 to exchange the initially bound GDP with GTP which leads to a conformational change of the GTPase. An N-terminal myristoyl anchor and an amphiphatic alpha helix are exposed allowing its stable membrane association (Antonny et al., 1997; Franco et al., 1996). A “priming complex” consisting of Arf1-GTP and members of the p24 family recruits in a next step the preassembled coatomer (Hara-Kuge et al., 1994) via interactions with its β-COP and γ-COP subunits (Zhao et al., 1997; Zhao et al., 1999). Together with recruited Arf GTPase activating protein (ArfGAP) (Eugster et al., 2000; Watson et al., 2004) the coatomer assembles into a lattice that concentrates cargo proteins with a cytosolic KKXX and KXKXX motif (X stands for any amino acid) (Cosson and Letourneur, 1994) into the nascent coated vesicle. KDEL receptors – transmembrane receptors important to recycle proteins from the early Golgi to the ER – can also bind the coatomer via a distinct dilysine motif in combination with a phosphorylated serine residue (Cabrera et al., 2003). Binding of the coatomer to the p24 proteins finally leads to a conformational change of the complex (Reinhard et al., 1999) that is likely to provide the energy to bend the membrane resulting in a coated COPI vesicle. Hydrolysis of GTP and the subsequent release of Arf1 from the membrane may act as a timer which triggers the release of the coat components and thereby prepares the vesicle for fusion (Tanigawa et al., 1993). Arf1-GTP hydrolysis is controlled by an ArfGAP (Cukierman et al., 1995) and the COPI complex which are both necessary for full GTPase activation (Goldberg, 1999).

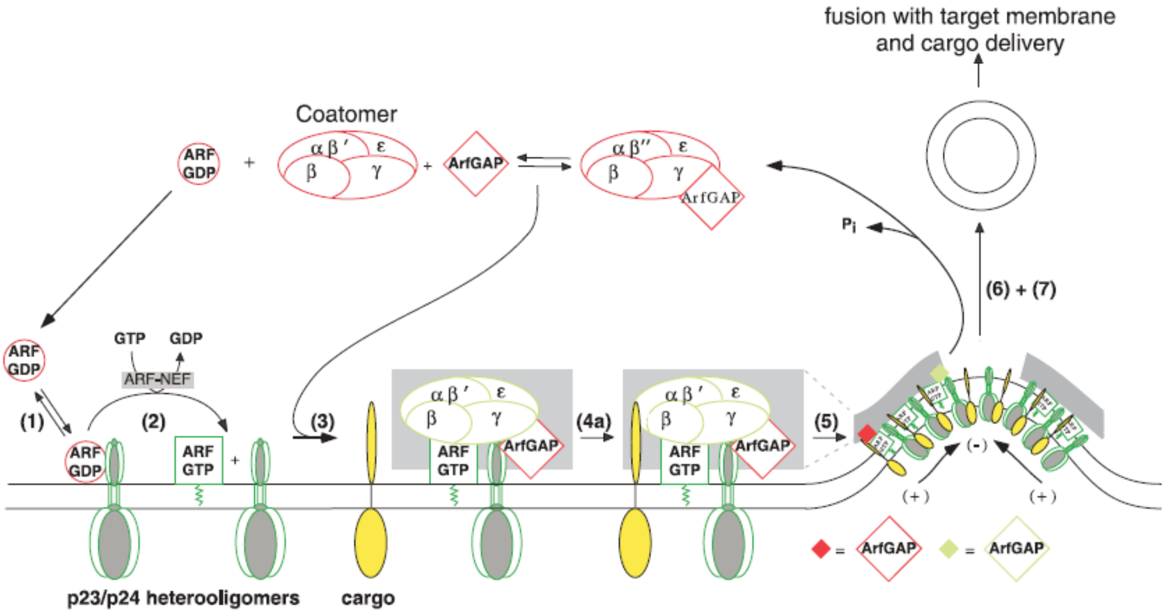

Figure 4. **COPI vesicle formation:**
Arf-GDP is recruited to the membrane by p23/p24 (1) and activated by a GEF (2). In a next step, the coatomer together with associated ArfGAP is recruited to the p24 oligomer and Arf-GTP (3). p24 proteins reduce the activity of ArfGAP, which allows the coatomer to capture other transmembrane cargo (4). After coat polymerization (5), the COPI vesicle can finally bud off the donor membrane (6), which occurs concominantly with ArfGAP-stimulated uncoating (7). Since ArfGAP activity is dependent on membrane curvature, there is a net coat dissociation at the tip of the nascent vesicle (-) and a net coat association at the rims (+) leading to a concentration of cargo in the forming vesicle. Active components are shown in green whereas inactive components are depicted in red. (Reproduced from Bethune et al., 2006).

For sorting of cargo into COPI vesicles two mutually non-exclusive models have been proposed. A combination of both models can be seen in Figure 4. It has been shown that the cytoplasmic tail of p23 and p24 inhibited ArfGAP1 mediated hydrolysis of Arf-GTP (Goldberg, 2000; Lanoix et al., 2001). A mechanism based on kinetic control of Arf1 mediated GTP hydrolysis was proposed. In the absence of specific cargo proteins, coatomer will rapidly dissociate from the membrane due to the high GTPase activity stimulated by noninhibited ArfGAP. In contrast, coatomer bound to Arf1 and for example p24 will be more stably associated to the membrane due to reduced ArfGAP stimulation. The priming complex would then have more time to diffuse on the membrane, capture other transmembrane proteins through coat affinity and build a lattice that would form a vesicle before uncoating occurs. However, this model does not explain the formation of COPI vesicles that do not contain p24 molecules, since other transmembrane proteins did not inhibit GTP hydrolysis of Arf 1.

The second model depends on the finding that ArfGAP1 activity is dependent on membrane curvature (Bigay et al., 2005; Bigay et al., 2003). According to this model there is a continuous flow of coat components from the rims of the nascent bud to its tip. At the tip there is high membrane curvature which induces ArfGAP1 activity so GTP is hydrolysed and the coat falls off. At the rim of the vesicle however, membrane curvature and consequently GTP hydrolysis would be lower. The released coat components from the tip would be replaced by the trimeric coatomer/Arf1/Arf1GAP complex from the rim. By this mechanism transmembrane cargo that binds directly to the coat would be sorted into the tip of nascent COPI vesicles.

2.2 COP II vesicles

In most eukaryotes, COPII vesicles are formed at specialized regions called ER exit sites (ERES) (Bannykh et al., 1996; Orci et al., 1991). They transport newly synthesized proteins and lipids from the ER to the ERGIC (Sato, 2004).

COPII vesicle formation begins with the activation of the small Ras-like GTPase Sar1 (Nakano and Muramatsu, 1989), mediated by the ER localized transmembrane GEF Sec12 (Barlowe and Schekman, 1993; Nakano et al., 1988) (Figure 5). The conversion of Sar1GDP to Sar1GTP leads to a conformational change which exposes an N terminal amphiphatic alpha helix that anchors Sar1 into the ER membrane (Bi et al., 2002; Huang et al., 2001). In a next step, activated Sar1 recruits the heterodimer Sec23/24 to form the so called “prebudding complex”. Sec23 directly interacts with Sar1GTP whereas the Sec24 subunit binds cargo proteins (Bi et al., 2002). There exist at least three independent signal binding sites on Sec24 that recognize distinct sorting signals on the cytosolic tails of transmembrane cargo. The best characterized signals contain a di-acidic ((D/E)X(D/E) (X = any amino acid) or dihydrophobic motif (FF,YY,LL,FY) (Dominguez et al., 1998; Nishimura and Balch, 1997).

It has been shown that many secretory proteins like VSV-G or ERGIC53 need to oligomerize to be efficiently recruited into COPII vesicles (Sato and Nakano, 2003). COPII may therefore recognize only sorting signals displayed in a specific assembly, achieved through oligomerization, which would ensure the efficient incorporation of fully assembled cargoes into COPII vesicles (Sato and Nakano, 2007). The prebudding complex subsequently recruits the outer layer of the COPII coat, the heterotetramer Sec13/31 (Lederkremer et al., 2001), which acts as a scaffold, like clathrin, and likely functions to cross-link adjacent prebudding complexes.

Membrane curvature is locally induced with the insertion of the N-terminal amphipathic alpha helix of Sar1 into the outer leaflet of the ER membrane (Lee et al., 2005). The concave inner surface of Sec23/24 is enriched in basic amino acids which are thought to bind to the acidic phospholipids that comprise the ER and therefore facilitate membrane bending (Bi et al., 2002) initiated by Sar1. The recruitment of Sec13/31, finally, is thought to propagate further curvature which finally leads to the formation of a vesicle. The exact mechanisms important for fission of COPII vesicles are not yet known, but it seems that Sar1 is an important factor in this event (Lee et al., 2005).

After budding of a COPII vesicle, uncoating has to take place before fusion with a target membrane, presumably because the docking and fusion machinery are buried within the coat. (Marcus c.s.lee 2007). It is thought that analogous to the COPI coat, GTP hydrolysis of Sar1 acts as a timer that triggers this event (Oka and Nakano, 1994). GTPase hydrolysis of Sar1 is controlled by Sec23 which acts as a GAP (Yoshihisa et al., 1993). This GAP activity is even stimulated further by the recruitment of Sec13/31 (Antonny et al., 2001). Therefore the assembly of the prebudding complex would trigger its own disassembly which may prevent COPII vesicle formation.

However, it has been shown in vitro, that Sec12 counteracts the GTPase stimulating activity by continually recharging Sar1 with GTP, so that Sec23/24 is maintained on the membrane (Futai et al., 2004). Furthermore, experiments with proteoliposomes demonstrated that Sec23/24 bound to cargo can remain transiently associated even after Sar1 GTP hydrolysis (Sato and Nakano, 2005). Thus a model was suggested where multiple low-affinity interactions of Sec23/24 with cargo signals stabilize the coat transiently after Sar1 dissociation until Sar1 is reactivated by Sec12. By this mechanism it is ensured that only COPII vesicles with proper cargo result in productive vesicles.

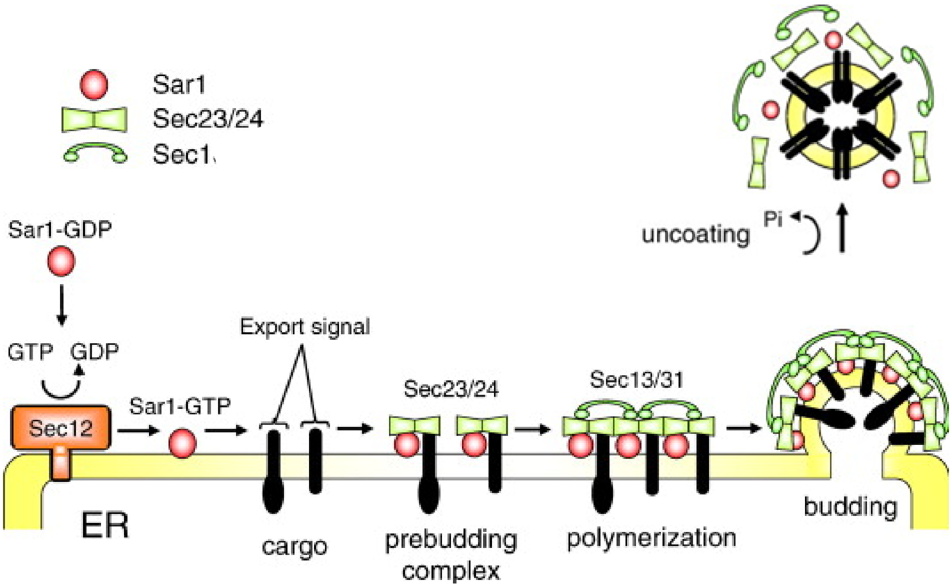

Figure 5. **COPII vesicle formation:**
Sar1 is activated by the transmembrane GEF Sec12 and thereby recruited to the ER membrane. Sec23/24 interacts with Sar1-GTP and binds to cytosolic sorting signals of cargo molecules forming the “prebudding complex”. Several of these complexes are clustered by Sec13/31 which finally leads to the formation of COPII coated vesicles. After budding, Sar1-GTP hydrolysis promotes vesicle uncoating allowing fusion with an acceptor membrane. (Adapted from Sato and Nakano, 2007).

2.3 Clathrin coated vesicles

Clathrin-coated vesicles (CCVs) mediate cargo transport at the plasma membrane, the TGN and endosomes. They were the first coated vesicles to be discovered (Pearse, 1976) and are therefore the best characterized class of transport carriers. It has been reported that more than 150 different proteins are associated with CCVs (Blondeau et al., 2004). The main component of these vesicles is clathrin which forms a polymeric mechanical scaffold that is linked to the membrane by an inner layer of clathrin adaptors. The formation of CCVs occurs within minutes. It is a highly regulated process and requires the perfect interplay of several adaptors, cargo receptors and accessory proteins.

2.3.1 Clathrin

The assembly unit of the clathrin coat is the triskelion, a clathrin trimer consisting of three heavy chains that trimerize through a C-terminal helical tripod arrangement (Fotin et al., 2004) and three light chains (Figure 6). The heavy chain can be subdivided into a C-terminal proximal domain at the trimerization zone, a distal domain which forms the typical knee, and a globular N-terminal domain. Triskelions can assemble into regular polyhedral structures of different shapes and sizes (Crowther et al., 1976) through interactions involving the distal and proximal leg regions of adjacent trimers. The flexible clathrin knee and the alteration of the angle at which the proximal domains cross are thought to be important for the adaptation of the lattice to different shapes and sizes (Fotin et al., 2004; Musacchio et al., 1999). The two clathrin light chain isoforms LCa and LCb that exist in higher eukaryotes were shown to bind to the proximal domain of the heavy chains and to reside outside of the lattice (Fotin et al., 2004). However, the exact role of both isomers is still unclear, but may be regulatory.

Purified clathrin triskelia can self-assemble into clathrin cages at low pH (Keen et al., 1979). However, adaptor proteins (see below) are absolutely required under physiological conditions to form a membrane coat since clathrin does not interact directly with a lipid bilayer (Kirchhausen, 2000; Lindner and Ungewickell, 1991; Vigers et al., 1986). The interaction with the adaptor proteins is mediated by the clathrin N-terminal domain that projects towards the centre. It forms a seven-bladed β-propeller which has three potential protein binding sites. It was shown to bind the W box motif (PWXXW, where X is any amino acid) and the clathrin box motif (LϕXΦ [D/E], where Φ is a bulky hydrophobic amino acid) that are present on many adaptor proteins and accessory factors.

In addition to its involvement in intracellular trafficking, clathrin seems also to play a role in other intracellular processes: It was demonstrated that the heavy chain binds to the mitotic spindle (Royle et al., 2005), and it was proposed that clathrin helps to regulate the segregation of chromosomes during mitosis. Additionally, clathrin depletion experiments prevented the efficient reassembly of the Golgi apparatus suggesting a role for clathrin in this process (Radulescu et al., 2007).

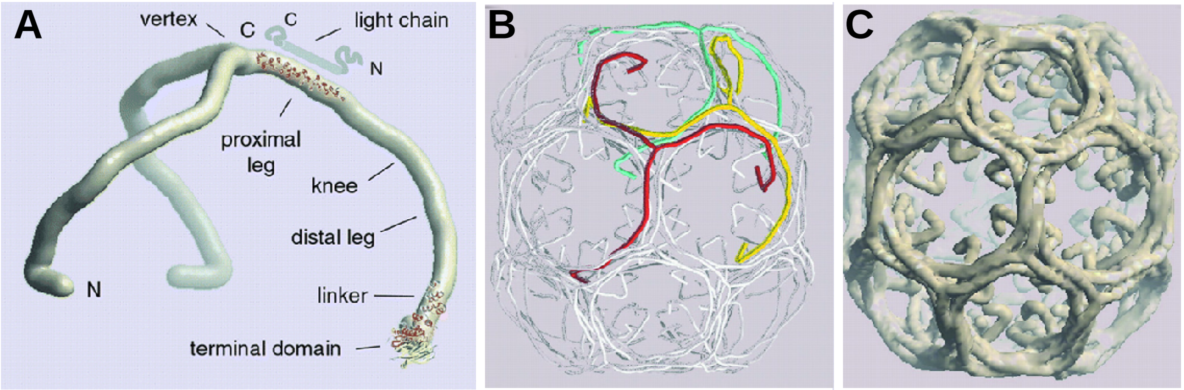

Figure 6. **Structure of a clathrin triskelion and its assembly into a clathrin coated vesicle.**
(A) Domain organization of a single clathrin triskelion (B) A clathrin barrel with three different triskelia highlighted in red, yellow and green. For simplicity the light chains have been removed. The N-terminal domains, face inward towards the membrane and are in close contact with the adaptors (not shown). (C) A clathrin cage reconstruction obtained by electron cryomicroscopy at ~21-Å resolution (Adapted from Kirchhausen, 2000).

2.3.2 Adaptor proteins

A clathrin adaptor is defined as a protein that links the clathrin scaffold to a component of the membrane, be it a phospolipid, a transmembrane receptor, or both together (Edeling et al., 2006). Over twenty different clathrin adaptors have been identified so far (Owen et al., 2004). All of them share two common characteristics: they bind to the N-terminal seven-bladed β-propeller of the clathrin heavy chain, and they share a common design principle in that they consist of compact folded domains with unstructured flexible linkers.

The first clathrin adaptors to be discovered were the heterotetrameric adaptor proteins AP-1 and AP-2. Since then two additional members of the AP family, AP-3 and AP-4 were discovered. They all consist of two large subunits of ~100kD (γ and β_1 in AP-1, α and β_2 in AP-2, δ and β_3 in AP-3, ε and β_4 in AP-4), a medium subunit of ~50kD (μ_{1-4}), and a small subunit of ~20kD (σ_{1-4}), which are assembled to form the typical structure of the AP family members with a core and two appendages connected via flexible linkers (Figure 7). All four APs bind directly to cytosolic sorting signals containing the YXXΦ (X represents any amino acid and Φ a large hydrophobic one) or the (D/E)XXXL(L/I) motif, although each heterotetramer does display individual preferences for particular residues at the X and Φ positions (Bonifacino and Glick, 2004; Ohno et al., 1998; Owen et al., 2004).

The APs, especially AP-1 and AP-2, are the best studied clathrin adaptors so far. More recently different alternative adaptors called clathrin-associated sorting proteins (CLASPs) were identified.

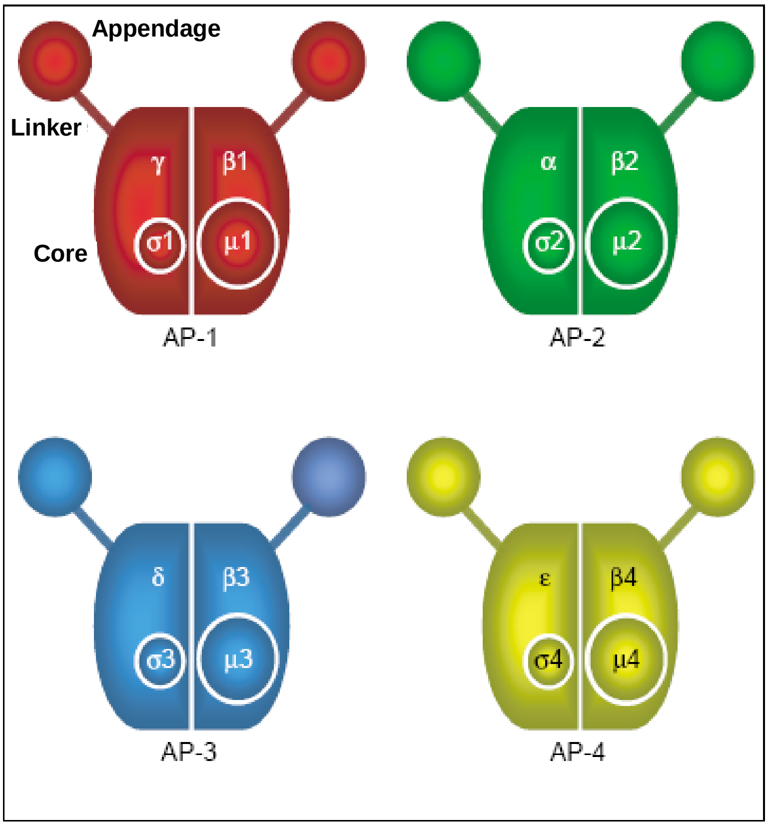

Figure 7. **Schematic representation of the four adaptor protein complexes:**
Each of the four APs consists of two large, a medium, and a small subunit. These are assembled into the typical AP structure of a core and two appendage domains connected via flexible linkers. For simplicity no isoforms of the different subunits are depicted in the figure. (Adapted from Robinson and Bonifacino, 2001).

AP-1

AP-1A is ubiquitously expressed and plays a major role in the assembly of CCVs at the TGN and endosomes. The expression of its isoform, AP-1B, on the other hand is restricted to polarized epithelial cells (Ohno et al., 1999) where it is involved in basolateral sorting of cargo (Folsch et al., 2003) at recycling endosomes (Cancino et al., 2007). The two isoforms differ only in their μ_1 subunit (Ohno et al., 1999). Nevertheless, they seem to have largely nonoverlapping functions in polarized cells.

Membrane recruitment of AP-1 is thought to be mediated through several low affinity interactions with activated Arf1, phosphatidylinositol (4)-monophosphate (PtdIns(4)P), sorting signals and a potential docking factor (see below). The interaction with PtdIns(4)P was demonstrated to be performed by the γ subunit, whereas Arf1 binding needed the trunk regions of both, γ and β_1 adaptin (Austin et al., 2000; Heldwein et al., 2004). AP-1 recognizes two different types of sorting signals. With its μ_1 subunit it binds the YXXΦ motif, which is one of the most common sorting signals in the cytosolic domains of transmembrane proteins (Bremnes et al., 1998; Ohno et al., 1995). Proteins such as the CI (cation independent) and CD (cation dependent) mannose 6-phosphate receptors (MPRs), LAMP1 and Furin were shown to interact with AP-1 via this sorting motif (Bonifacino and Traub, 2003). The other signal, (D/E)XXXL(L/I), was recently shown to be recognizes by the γ/σ_1 hemicomplex (Doray et al., 2007) on proteins like the lysosomal transmembrane protein LIMPII (Fujita et al., 1999).

Membrane recruitment of AP-1 is a highly regulated process. A 4Å resolution crystal structure of the AP-1 revealed that the μ_1 subunit adopts a closed conformation in the cytosol, so that the tyrosine motif binding site is not accessible (Heldwein et al., 2004). It is thought that upon phosphorylation, μ_1 performs a conformational change resulting in the exposure of its signal binding motif. Furthermore it was shown that β_1 phosphorylation inhibits clathrin binding to its linker domain, (Wilde and Brodsky, 1996) supporting a role of phosphorylation in regulating AP-1 and clathrin recruitment (Ghosh and Kornfeld, 2003a).

It has become uncertain where exactly AP-1 functions. Originally it was thought that AP-1A is involved in sorting cargo into TGN derived CCVs (Doray et al., 2002; Zhu et al., 1999a). However, there are other studies that provide evidence for a function of AP-1A at early endosomes where it is important for recycling cargo to the PM (Pagano et al., 2004). Finally there are reports which show the involvement of AP-1A in retrograde transport of cargo from endosomes to the TGN (Meyer et al., 2000).

AP-2

Many studies have shown that AP-2 is the adaptor involved in clathrin mediated endocytosis at the plasma membrane. In mammals there exist two different α-subunit isoforms, but no functional difference has been reported so far (Ball et al., 1995). AP-2 is targeted to the plasma membrane mainly through its interaction with phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) and/or phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) mediated by the α-subunit (Gaidarov et al., 1996; Gaidarov and Keen, 1999; Rohde et al., 2002). A mutation of the α-subunit which abolishes binding to PtdIns(4,5)P_2 was shown to prevent AP-2 recruitment even in the presence of sorting signals (Honing et al., 2005). However, AP-2 seems also to have an additional PtdIns(4,5)P_2 binding site in its μ_2 subunit. (Collins et al., 2002). Membrane localisation of AP-2 at the plasma membrane is brefeldin A (BFA) insensitive. BFA inhibits the activation and therefore also the resulting membrane binding of the small GTPases Arf1-5, thus in contrast to AP-1, AP-2 does not need Arf1 for its membrane recruitment. However, Arf6 remains a possible candidate (Paleotti et al., 2005), because its membrane localization is not sensitive to BFA treatment.

The μ_2 subunit of AP-2 recognizes cytosolic sorting signals of the FXNPXY and YXXΦ motif (Ohno et al., 1995) on cargo like the transferrin receptor or TGN38 (Boll et al., 2002). In addition the α/σ_2 hemicomplex has been identified to mediate cargo binding to proteins containing the (D/E)XXXL(L/I) motif (Doray et al., 2007).

Similar to AP-1, AP-2 recruitment is highly regulated. Recently, the regulatory mechanism of YXXΦ signal recognition of μ_2 has been revealed (Nakatsu and Ohno, 2003; Owen et al., 2004; Traub, 2005). In the cytosol, μ_2 is in a closed conformational state similar to μ_1. Upon its phosphorylation at Thr156 by adaptor associated kinase 1 (AAK1) (Conner and Schmid, 2002), μ_2 makes a conformational change to expose its tyrosine signal binding site. AAK1 is associated with AP-2 like many other regulatory/accessory proteins via binding to the α-appendage (Owen et al., 1999; Owen et al., 2000). Its kinase activity was recently shown to be stimulated by clathrin, upon binding to the β_2 linker domain of AP-2 (Conner and Schmid, 2003; Jackson et al., 2003).

AP-3

The first clathrin adaptors discovered were AP-1 and AP-2. Soon it became clear that these two proteins could not be sufficient to perform all the sorting events involving (D/E)XXXL(L/I) and YXXΦ signals. By searching sequence databases for homologues of AP-1 and AP-2, the existence of two other members of the AP family, AP-3 and AP-4 was soon revealed.

There exist two isoforms of the β_3, μ_3 and σ_3 subunit. β_{3B}, and μ_{3B} are part of an AP-3 isoform that is only expressed in neuronal and neuroendocrine cells. All other isoforms are ubiquitously expressed (Robinson and Bonifacino, 2001). Studies making use of naturally occurring AP-3 mutants in flies, mice and humans have shown that the ubiquitously expressed AP-3 functions in transport pathways en route to lysosomes or related organelles like melanosomes (Dell'Angelica et al., 1999; Kantheti et al., 1998). The neuronal specific isoform of AP-3 on the other hand, seems to play a critical role in the formation and function of a subset of synaptic vesicles (Seong et al., 2005).

As in the case of AP-1, also AP-3 recruitment to membranes was shown to be Arf1 dependent (Ooi et al., 1998). At the membrane it recognizes YXXΦ motifs in cargo like LAMP1 with its μ_3 subunit (Ohno et al., 1998), whereas the δ /σ_3 hemicomplex seems to be involved in cargo recognition of the (D/E)XXXL(L/I) motif present on cargo like tyrosinase (Janvier et al., 2003). AP-3 is not enriched in CCVs (Simpson et al., 1996) and it might even function in a clathrin independent manner in some circumstances (Peden et al., 2002). Nevertheless it was shown that both β_3 subunits can bind clathrin in vitro, and in a recent report, clathrin depletion inhibited the sorting of AP-3 dependent cargo proteins (Chapuy et al., 2008), thus it seems that AP-3 generates CCVs.

The exact organelle at which AP-3 is functioning is still a matter of debate. Originally it was thought that AP-3 mediates cargo sorting at the Golgi, but more recent data suggest that it mediates sorting at endosomes (Peden et al., 2004).

AP-4

AP-4 is the newest and least studied adaptor of the AP family. It consists of 2 large (ε, β_4), a middle (μ_4) and a small subunit (σ_4). However, AP-4 was shown to be phylogenetically different from the other three family members. A direct comparison revealed that it lacks an appendage in the β_4 subunit and therefore can not bind clathrin (Lundmark and Carlsson, 2002). Nevertheless it was reported that AP-4 is involved in clathrin mediated sorting of CI-MPR at the TGN (Barois and Bakke, 2005), maybe via a potential clathrin binding site in the ε subunit.

AP-4 is associated with the TGN and/or endosomes (Barois and Bakke, 2005) where it is involved in several sorting processes. In MDCK cells, disruption of AP-4 led to a nonselective transport to both the apical and basolateral domains of basolateral proteins such as the low density lipoprotein receptor (LDLR) (Simmen et al., 2002). Therefore AP-4 seems to play a role in basolateral sorting. In other experiments μ_4 was shown to interact with the YXXΦ motif of lysosomal cargo such as LAMP2 and mediate their direct transport to lysosomes (Aguilar et al., 2001). Finally, neuronal AP-4 seems to be important in regulating proper somatodentritic distribution of cargo proteins (Matsuda et al., 2008).

Not much is known about the recruitment of AP-4 to membranes. It was shown that it needs Arf1 (Boehm et al., 2001), but more work has to be done to reveal the regulation of AP-4 recruitment and to determine its exact trafficking route.

CLASPs (clathrin-associated sorting proteins)

AP-2 does not recognize all sorting signals that lead to clathrin dependent endocytosis. Other signals like ligand induced phosphorylation or ubiquitylation, do not use AP-2 as the principal sorting adaptor. When cultured cells were transfected with siRNA oligonucleotides directed against AP-2 there was no significant reduction in EGF or LDL receptor internalization although the same receptors were not taken up after siRNA mediated silencing of clathrin (Hinrichsen et al., 2003; Huang et al., 2004; Motley et al., 2003).

Thus there have to exist other adaptors that can sort these cargoes into clathrin coated vesicles, even in the absence of AP-2. However, RNAi-mediated silencing of AP-2 protein levels also diminished the number of clathrin coated strucutres at the cell surface more than tenfold, showing that under physiological conditions AP-2 acts together with these other adaptors to sort cargo into forming CCVs.

The appendage domains of AP-1 and AP-2 were shown to bind to different groups of so called clathrin-associated sorting proteins (CLASPs) and it was proposed that these CLASPs recognize cargo with distinct sorting signals and target it into forming CCVs through the interaction with AP-1 or AP-2. Therefore, CLASPs can account for the concentration of numerous different cargo types within a single coat (Figure 8A).

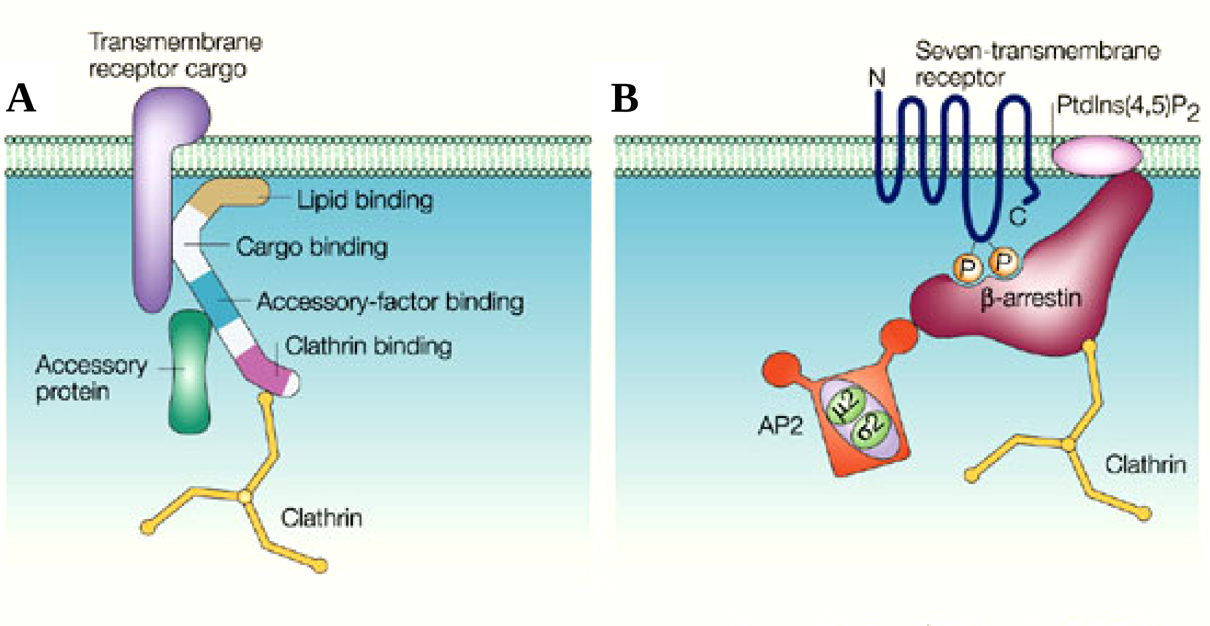

Figure 8. **Clathrin-associated sorting proteins (CLASPs) at the plasma membrane.**
(A) Domain organization of a typical CLASP (B) The CLASP β-arrestin and its interaction partners. (Adapted from Wendland, 2002).

β-Arrestins: β-arrestin 1 and 2 are the founding members of the CLASP family (Figure 8B). They consist of two domains made of β-sheets. The C-terminal domain seems to be involved in PtdIns(4,5)P_2 binding, (Gaidarov and Keen, 1999; Milano et al., 2002) whereas the N-terminal domain recognizes the large family of G-protein coupled receptors (GPCR) (Han et al., 2001; Milano et al., 2002; Oakley et al., 2001; Pulvermuller et al., 2000). Binding of the phosphorylated, ligand activated GPCRs induces a conformational change in the β-arrestins (Schleicher et al., 1989) that leads to the release of a C-terminal fragment containing binding sites for clathrin and the β_2 subunit of AP-2. This allows β arrestins to target bound GPCRs to CCVs for endocytosis (Lefkowitz and Whalen, 2004; Marchese et al., 2003). Thus, the task of promoting the internalization of the largest receptor family known (GPCR) is not assigned to AP-2 directly, but to a pair of monomeric CLASPs.

Dab2 and ARH: Studies of patients and knockout mice that fail to take up LDL have shown that Dab2 (disabled-2) and ARH (autosomal recessive hypercholesterolemia) proteins are involved in the uptake of members of the LDL receptor family (Eden et al., 2002; Garcia et al., 2001). Both adaptors contain an N-terminal PTP domain which recognizes the FXNPXY sorting motif found in the cytoplasmic tails of LDL receptors. Like the β-arrestins, Dab2 and ARH also contain a PtdIns(4,5)P_2 binding site and can interact with clathrin and the β_2 adaptin, which makes them prefect candidates to sort LDL receptors into forming CCVs at the plasma membrane (He et al., 2002; Mishra et al., 2002; Nagai et al., 2003).

GGA: CLASPs do not only act at the plasma membrane. In fact several CLASPs are known to be involved in sorting cargo in AP-1 containing vesicles. The most prominent of these CLASPs are the Golgi localized γ-ear containing, Arf-binding (GGA) proteins.

GGA proteins were initially discovered by searching databases for AP subunit homologs (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000). There exist three different GGAs in mammals (Bonifacino, 2004; Robinson, 2004). All of them are monomeric, ubiquitously expressed clathrin adaptors that consist of an N-terminal VHS domain, a GAT domain, and a C-terminal GAE domain which shows amino acid sequence homology to the γ-appendage of AP-1.

GGAs are found predominantly at the TGN and immuno fluorescence studies showed a reasonable colocalization with AP-1 (Hirst et al., 2000). Membrane localisation of the GGA proteins involves binding of activated Arf1 (Bonifacino, 2004) and PtdIns(4)P (Wang et al., 2007) via the GAT domain. At the membrane, the VHS domain recognizes DXXLL-type acidic clusters on cargo like CD- and CI-MPRs (Puertollano et al., 2001; Takatsu et al., 2001; Zhu et al., 2001). This interaction was shown to be strengthened upon phosphorylation of serine residues adjacent to the acidic cluster dileucine sequence. Even though GGAs contain clathrin boxes to directly interact with clathrin (Puertollano et al., 2001), they are absent from preparations of CCVs (Hirst et al., 2000). Because of this and the fact that GGA proteins where shown to bind the AP-1 γ-appendage (Bai et al., 2004), a model was proposed where GGA proteins sort cargo into AP-1 containing vesicles.

GGA1 and 3 contain an intramolecular DXXLL site (Doray et al., 2002). After cargo binding GGA proteins bind AP-1. Casein kinase II which is associated with AP-1 via the γ-appendage is then thought to phosphorylate the internal GGA-DXXLL sequence and thereby trigger the discharge of VHS from the cargo to the internal motif. After this, cargo would then be transferred to AP-1. Casein kinase II mediated phosphorylation of GGA1/3 also decreases their affinity for the γ–appendage, promoting their dissociation from AP-1 (Ghosh and Kornfeld, 2003b). This way, GGA1 and GGA3 could act as connector proteins to funnel cargo into coated buds assembling at the TGN (Doray et al., 2002). The fact that transfected CI-MPRs with a mutated DXXLL sorting signal were not properly sorted at the TGN strengthens this idea (Doray et al., 2002).

However GGAs have also been visualized on tubulo-vesicular MPR-positive structures exiting the TGN in live cell imaging experiments (Puertollano et al., 2001), which would speak against this model.

EpsinR: Another Golgi CLASP, EpsinR (epsin related protein), was identified in proteomic analysis of purified CCVs or γ-appendage binding partners (Hirst et al., 2003; Wasiak et al., 2002). It consists of an N-terminal ENTH domain that is followed by a long unfolded polypeptide chain. The ENTH domain of EpsinR shows homology to the one in epsin, (Hirst et al., 2003) a protein involved in endocytosis (see below) (Chen et al., 1998; Itoh et al., 2001; Overstreet et al., 2003). However there seems to be no overlap in intracellular localization of these two proteins.

The ENTH domain binds PtdIns(4)P and localizes EpsinR to the TGN/endosomes where it can interact with AP-1, clathrin and GGA2 through its unstructured C-terminal domain. EpsinR shows a reasonable colocalization with AP-1 in the juxtanuclear region and siRNA experiments demonstrated that neither of them is needed to recruit the other (Hirst et al., 2003). However, siRNA experiments in HeLa cells showed that the SNARE protein vti1b was mislocalized without AP-1 or EpsinR and a strong reduction of vti1b within CCVs was observed (Hirst et al., 2004). Thus it seems that EpsinR is a CLASP that selectively gathers SNARE proteins into AP-1 containing CCVs. Additionally, EpsinR seems to play a role in clathrin dependent retrograde traffic from recycling endosomes to the TGN (Saint-Pol et al., 2004).

The aftiphilin / γ-synergin / p200 complex: γ-synergin was the first γ-binding protein that was discovered (Page et al., 1999). In the cytosol, it assembles into a ternary complex with aftiphilin and p200 (Hirst et al., 2005). Aftiphilin is an unstructured protein, with multiple γ-appendage and two clathrin binding sites. p200 in contrast has no AP-1 binding sites and can only interact indirectly with AP-1. In cultured cells, a high colocalization of γ-synergin with AP-1 was observed. AP-1 silencing led to a cytosolic distribution of aftiphilin and γ-synergin

(Hirst et al., 2005; Lui et al., 2003). Thus it seems that AP-1 anchors the complex to the membrane.

siRNA experiments in HeLa cells against aftiphilin led to a redistribution of transferrin from recycling to sorting endosomes (Hirst et al., 2005). This implies that the aftiphilin/γ-synergin/p200 complex cooperates with AP-1 at early endosomes where it may help to sort certain SNARES that are important for fusion with the recycling endosome.

2.3.3 Accessory factors

More than twenty different accessory proteins were found to interact with the appendage domains of AP-2 (Lafer, 2002; Robinson, 2004; Traub, 2003). Most of them are essential in the formation of CCVs since they mediate processes like membrane deformation, vesicle fission or uncoating of the budded vesicle. Most of these accessory factors were not enriched in CCVs (Chen et al., 1998) which indicates that they interact only transiently with the coat.

Proteomic and bioinformatic analysis of γ-appendage interaction partners also identified several accessory factors for AP-1. However, much less is known about their role in CCV formation.

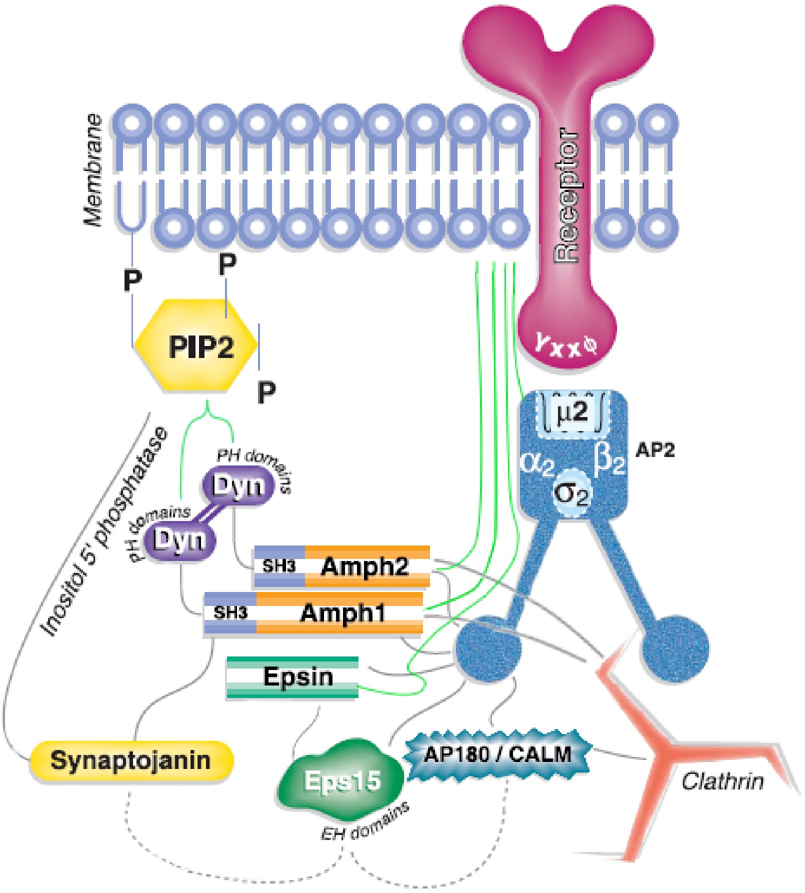

Figure 9. **Some of the accessory factors involved in CCV formation at the plasma membrane.** AP-2 recognizes specific sorting signals on transmembrane cargo. Different accessory factors interact with each other and with the appendage domain of AP-2 whereas clathrin binds at the linker region. Green lines depict protein-lipid interactions. The dashed lines indicate interactions that may not occur for neuronal forms of synaptojanin and AP180. (Adapted from Marsh and McMahon, 1999).

EPS15: Epidermal growth factor protein substrate 15 (EPS15) is enriched at synapses (Chen et al., 1998) and interacts with the appendage domains of AP-2 (Salcini et al., 1999) through several DFP motifs at its C-terminus. The N-terminal region consists of three EH domains and binds to several endocytic proteins including epsin 1 (Figure 9). It was shown that EPS15 localizes to the edges of clathrin coated pits (Tebar et al., 1996) where it might function at the periphery of the coat. Overexpression studies with the C-terminal domain of EPS15 inhibited the clathrin mediated uptake of the transferrin and epidermal growth factor receptor demonstrating the requirement of EPS15 for receptor mediated endocytosis (Benmerah et al., 1998). Interestingely a very recent paper demonstrated that EPS15 also interacts with AP-1 at the TGN (Chi et al., 2008). Disruption of this interaction via overexpression of a mutant EPS15 lacking the AP-1 interacting site significantly reduced the exit of secretory cargo from the TGN.

Epsin 1: Epsin 1 belongs to the epsin family consisting of four members: epsin 1-3 and the already described epsinR. It is enriched in neuronal cells (Chen et al., 1998; Rosenthal et al., 1999) and it is the main binding partner for EPS15 (Figure 9). It has a highly conserved ENTH domain at the N-terminus which is important for membrane binding. The following unfolded polypeptide chain on the other hand was shown to be essential for its interaction with AP-2 and clathrin.

Expression of epsin fragments and microinjection of antibodies against epsin 1 inhibited clathrin mediated endocytosis (Chen et al., 1998; Rosenthal et al., 1999). Although the exact role of epsin 1 is still unclear, in vitro and in vivo experiments showing that the ENTH domain is able to induce membrane curvature (Ford et al., 2002) indicate its involvement in membrane deformation.

Furthermore, it was shown that epsin 1 and EPS15 bind ubiquitinated cargo with their ubiquitin interacting motifs and sort it into CCVs (Hoeller et al., 2006; Polo et al., 2002; Sigismund et al., 2005; Traub and Lukacs, 2007). Therefore they might also have a role as CLASPs in sorting ubiquitinated proteins like the activated EGFR.

AP180/CALM: AP180 is a brain specific protein that is enriched in purified CCVs. Its functional homolog, CALM (clathrin assembly lymphoid myeloid leukaemia), is ubiquitously expressed (Tebar et al., 1999). AP180 is recruited to the plasma membrane via the interaction with PtdIns(4,5)P_2 through its N-terminal ANTH domain (Ford et al., 2001). At the membrane AP180 can interact with clathrin and the two AP-2 appendage domains (Owen et al., 1999; Owen et al., 2000). It was shown that the complex of AP180 together with AP-2 had a much stronger ability to assemble clathrin than each protein alone (Hao et al., 1999) indicating a role of AP-180 in CCV formation. Additionally it was proposed that AP180 mediates control of vesicle size (Ye and Lafer, 1995), since clathrin coats assembled in the presence of AP180 were smaller and more homogenous in size than coats formed in its absence. These results were later confirmed in vivo. Disruption of the gene encoding the AP180 homolog in Drosophila led to both fewer and larger CCVs (Zhang et al., 1998).

Synaptojanin: Synaptojanin is a major inositol-5-phosphatase in the brain where it colocalizes significantly with clathrin associated components (McPherson et al., 1996). In mammals, there exist two isoforms which undergo alternative splicing to generate multiple isoforms that display a different tissue and subcellular distribution. They all consist of two inositol phosphatase domains, followed by a proline-rich domain that mediates SH3 domain binding (McPherson et al., 1996) with endophilin or the ampiphysin1/2 complex (Figure 9). The main substrate of synaptojanin is plasma membrane enriched PtdIns(4,5)P_2 which it converts to phosphatidylinositol. AP-2 is targeted to the membrane mainly through the interaction with PtdIns(4,5)P_2. Consequently synaptojanin is thought to promote membrane dissociation of AP-2, probably during the uncoating process. Additionally it was proposed that synaptojanin might regulate dynamin activity, either through its competitive binding to the known dynamin interactors endophilin and amphiphysin1/2, or through the dephosphorylation of PtdIns(4,5)P_2 and the subsequent release of dynamin from the membrane. The importance of synaptojanin in vesicle fission was shown in knockout mice that showed deeply invaginated clathrin coated pits unable to perform scission (Kim et al., 2002).

Amphiphysin 1: Amphiphysin 1 is predominantly expressed in the brain where it is localized in the cytosol and at presynaptic terminals (Wigge et al., 1997a). The N-terminus of amphiphysin 1 features a short amphipathic alpha helix, followed by a BAR domain, a central insert domain, and a C-terminal SH3 domain. The BAR domain mediates homo- or heterodimerization with amphiphysin 2 (Figure 9) (Slepnev et al., 1998) which results in a curved (banana-shaped) BAR domain dimer that is perfectly designed to sense and bind curved membranes with its positive charges on the concave surface. Since purified amphiphysin 1 was shown to invaginate liposomes into narrow tubules (Takei et al., 1999), it is thought that it not only senses curved membranes, but also induces further curvature. Clathrin heavy chain as well as the AP-2 α-appendage were shown to bind the central region of amphiphysin 1 through two distinct but partly overlapping sites (Slepnev et al., 2000). Additionally, also endophilin, another member of the BAR family interacts with this region, while synaptojanin and dynamin bind amphiphysin 1 through its C-terminal SH3 domain.

All these interactions support a model where amphiphysin 1 recruits synaptojanin and dynamin to the curved neck of forming CCVs and thereby supports vesicle fission and uncoating. This was confirmed by microinjection experiments: an SH3 domain of amphiphysin 1 was injected into the giant reticulospinal synapse of the lamprey eel which resulted in a dramatic accumulation of deeply invaginated clathrin coated pits at the plasma membrane. The reported stimulation of the GTPase activity of dynamin by amphiphysin 1 on membranes, further supports this model (Yoshida et al., 2004).

Surprisingly, amphiphysin 1 knockout mice showed only mild defects in endocytosis that only became apparent under conditions of strong nerve stimulation (Di Paolo et al., 2002). No accumulation of coated invaginations at the plasmalemma could be detected. The reason for this discrepancy between results obtained from microinjection experiments in cultured cells and the results obtained by gene knockout in mice is not yet clear.

Amphiphysin 2: Vertebrates contain an additional isoform of amphiphysin 1 which was identified by several groups resulting in its having different names e.g. amphiphysin 2, Amph2, BRAMP2, ALP1, SH3P9 and Bin 1. Here, I will refer to it as amphiphysin 2.

There exist at least 10 different splice variants of amphiphysin 2 which have different tissue and intracellular distributions (Tsutsui et al., 1997; Wigge et al., 1997a). The longest splice variant is brain specific and exhibits 71% amino acid sequence similarity and 55% amino acid sequence identity with amphiphysin 1 (Butler et al., 1997). Like amphiphysin 1 it consists of an N-terminal BAR domain followed by a central insert domain and a C-terminal SH3 domain. In vitro experiments showed its ability to form homo- or heterooligomers with amphiphysin 1 via its BAR domain (Figure 9). An N-terminal insert domain (NTID) within the BAR domain seems to contribute to both dimerization and membrane binding. Like amphiphysin 1, amphiphysin 2 also binds to endophilin (Micheva et al., 1997), AP-2 (Leprince et al., 1997; McMahon et al., 1997) and clathrin, (McMahon et al., 1997) via the central insert region and synaptojanin and dynamin with the SH3 domain. However, although both amphiphysins can bind synaptojanin and dynamin, competition experiments suggest that the two SH3 domains may differ in their affinity for these proteins (Butler et al., 1997; Slepnev et al., 1998; Wigge et al., 1997b). Amphiphysin 1 knockout mice were devoid of expressed amphiphysin 2, therefore it was suggested that amphiphysin 2 is only stable in a complex with amphiphysin 1. Amphiphysin 1 expression, however, was not dependent on amphiphysin 2.

Interestingly, a recent report has shown that amphiphysin 2 also interacts with sorting nexin 4 via its BAR domain. A pool of amphiphysin 2 colocalized with sorting nexin 4 at early endosomes (Leprince et al., 2003). Thus amphiphysin 2 may also have a role on endosomes.

Endophilin: The five isoforms of endophilin (A1, A2, A3, and B1 and B2) all belong to the BAR family and have a similar structure to amphiphysin 1. However, the central insert domain of amphiphysin is missing, thus endophilin can not interact with clathrin or AP-2.

The N-terminal BAR domain mediates dimerization and membrane binding. Initially it was thought that it also had a lysophosphatidic acid acyl transferase activity (Schmidt et al., 1999), but a recent study found this activity to be due to a contaminant (Gallop et al., 2005). The C-terminal SH3 domain interacts with synaptojanin, dynamin, amphiphysin 1 and amphiphysin 2. Even though the brain enriched endophilin A1 seems to be able to bind to both synaptojanin and dynamin, the affinity for the former is considerably greater, making it the preferred binding partner for synaptojanin (de Heuvel et al., 1997).

Functionally, endophilin was shown to be important for synaptic endocytosis. Microinjection of its SH3 domain produced a block in vesicle fission and the accumulation of clathrin coated vesicles, suggesting a dual role in membrane fission and uncoating (Gad et al., 2000). The exact role of endophilin, however, is still unclear.

Dynamin: Dynamin is a large GTPase involved in vesicle scission of clathrin coated and other endocytotic vesicles. There exists three dynamin isoforms, each with numerous splice variants, but only the neuronal specific dynamin1 and the ubiquitously expressed dynamin 2 were shown to play a role in endocytosis (Altschuler et al., 1998; Cao et al., 1998; Vallee and Shpetner, 1993). Dynamin consists of an N-terminal GTPase domain followed by a pleckstrin homology domain (PH) and a C-terminal proline rich domain (PRD).

It was demonstrated that the PRD of dynamin interacts with the SH3 domains of endophilin and amphiphysin. Through these interactions dynamin is thought to be recruited to the membrane where it can interact with PtdIns(4,5)P_2 via its PH domain (Figure 9) (Achiriloaie et al., 1999). At the membrane, dynamin forms rings around the neck of coated pits (Takei et al., 1995) and it is thought that GTP hydrolysis correlates with a conformational change in dynamin which pinches off the vesicle by constriction (Hinshaw and Schmid, 1995; Takei et al., 1995). Interestingly, dynamin was also shown to modulate the activation of the Arp 2/3 actin filament nucleation complex, and thereby de novo actin filament assembly at the cortex (Kessels and Qualmann, 2004; Krueger et al., 2003). A similar role has been proposed for sorting nexin 9 (see below). The mechanical force that is generated by de novo actin filament assembly may support the dynamin mediated scission of vesicles. The exact mechanism of action, however, is still not clear.

Sorting nexin 9: Sorting nexin 9 (SNX9) belongs to the SNX-BAR subfamily. Like all sorting nexins it contains a phox-homology domain (PX) (Teasdale et al., 2001) which in the case of sortin nexin 9 interacts with PtdIns(4,5)P_2. The C-terminal BAR domain on the other hand is important for dimerization and for binding to positively curved membranes. Through the combination of these two important domains, SNX9 is recruited to high-curvature, PtdIns(4,5)P_2-containing subdomains of the plasma membrane (Yarar et al., 2007), where it interacts with clathrin, AP-2, dynamin and the actin related protein-2/3 (Arp2/3) complex activator N-WASP (Badour et al., 2007; Lundmark and Carlsson, 2004; Soulet et al., 2005).

SNX9 was shown to be essential in the late stages of clathrin mediated endocytosis (Yarar et al., 2007). Its exact mechanism of action is unknown, but a model was proposed where SNX9 is recruited to forming CCVs at the plasma membrane, where it oligomerizes and thereby drives vesicle tubulation and clustering of N-WASP. The recruitment of N-WASP leads to an Arp2/3 mediated nucleation of actin filament assembly which generates enough force to help dynamin in the final fission process (Figure 10) (Yarar et al., 2007).

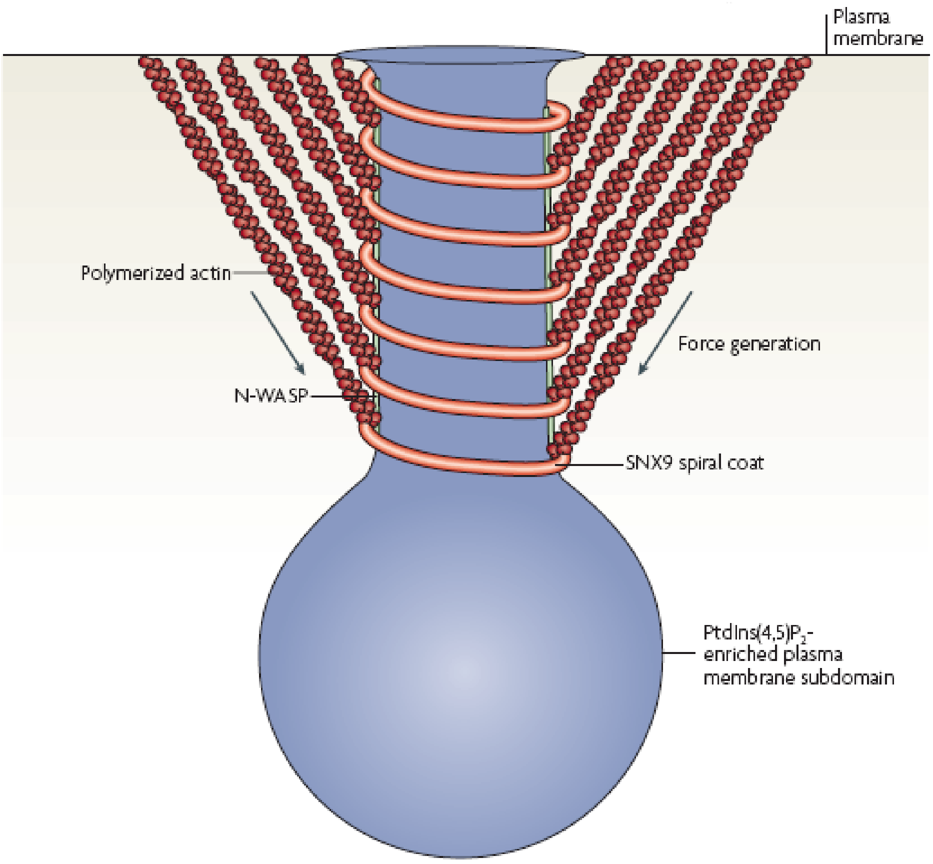

Figure 10: **The role of sorting nexin 9 (SNX9) in plasma membrane remodelling and membrane scission.** For simplicity the interaction of SNX9 with dynamin is not shown in the figure. (Reproduced from Cullen, 2008).

Auxilin: Once a clathrin coated vesicle is formed, the coat is efficiently removed to allow the vesicle to fuse with its target membrane and deliver its cargo. The uncoating machinery consists of auxilin and the heat shock cognate 70kD protein (Hsc70). There exist two auxilin isoforms, the neuronal specific auxilin1 and the ubiquitously expressed auxilin 2, also known as cycling G associated kinase (GAK). Auxilin binds to AP-2 and clathrin and thereby, recruits Hsc70 via its C-terminal J domain to the assembled clathrin coat (Umeda et al., 2000; Ungewickell et al., 1995). At the membrane the J domain is thought to activate the ATPase activity of Hsc70, which ultimately leads to uncoating of the vesicle (Barouch et al., 1997). The exact mechanism of this process is still not known, but it is proposed that in a first step auxilin induces a conformational change in the clathrin coat, allowing Hsc70 to bind. This would then destabilize the numerous interactions of the clathrin triskelia and promote clathrin release from the vesicle (Fotin et al., 2004). However, to release the adaptor layer, Hsc70 was shown to need an additional factor (Hannan et al., 1998). Ghosh and Kornfeld could demonstrate that Hsc70 interacts with the protein phosphatase 2A (PP2A), a protein that was shown to dephosphorylate the μ_1 subunit of AP-1 (Ghosh and Kornfeld, 2003a). When CCVs were incubated with ATP, purified Hsc70, and PP2A, AP-1 was released from the vesicles. Interestingly also AP-2 was released under these conditions. Therefore PP2A seems to be the cofactor of Hsc70 involved in the release of the adaptor layer. Additionally, synaptojanin was shown to dephosphorylate membrane lipids, reducing the membrane affinity of the adaptors even more (Verstreken et al., 2003).

2.4 Alternative transport vesicles

2.4.1 Caveolae

Caveolae are cholesterol and spingolipid-rich plasma membrane invaginations involved in clathrin independent endocytosis, transcytosis and signal transduction (Anderson, 1998; Parton and Simons, 2007). They contain characteristic signalling molecules and cargo receptors and were shown to be the primary carriers in endothelial cells. The major structural protein in caveolae is the ubiquitously expressed integral membrane protein caveolin 1 (Stan, 2005). There exist also two other isoforms with different splice variants: ubiquitously expressed caveolin 2, which seems to have a role in regulation of vesicle size, and muscle specific caveolin 3 (Scherer et al., 1995; Tang et al., 1996).

Caveolar internalization is a highly regulated process and not fully understood. Several tyrosine kinases including Src, but also molecules like EGF were shown to promote tyrosine phosphorylation of caveolin 1 (Lee et al., 2000; Li et al., 1996), which seems to be essential for caveolae mediated endocytosis (Sverdlov et al., 2007). However, the exact role of this phosphorylation event remains unclear. A model was proposed where the clustering of various cargo receptors initiates endocytosis via caveolae (Figure 11). In this model, caveolin 1 and 2 form heterooligomers that serve as a scaffold for cargo receptors and stabilize caveolae at the membrane. By an unknown mechanism, ligand binding and subsequent clustering of the cargo receptors leads to the activation of several tyrosine kinases that phosphorylate caveolin 1 and 2, as well as dynamin. Phosphorylation of caveolin 1 and 2 leads to a lateral dissociation of caveolin 2 from the coat and it is thought that it subsequently participates in the fission process. Phosphorylation of dynamin, on the other hand, was shown to increase its GTPase activity (Ahn et al., 2002; Ahn et al., 1999) and to promote its association with caveolin 1 at the neck of the caveolae, where it finally mediates fission of the vesicle (Kim and Bertics, 2002). The coated vesicle travels along microtubules (Tagawa et al., 2005) to organelles like the caveosome which serves as an intermediate station during internalization of ligands in the caveolar endocytic pathway. In contrast to COPI/II, or clathrin coats, caveolin, as a membrane protein, is not disassembled after budding of the transport carrier. It follows the vesicle from the donor to the acceptor domain. Therefore caveolae are also present in the membranes of caveosomes, early endosomes and TGN (Nichols, 2002; Pelkmans et al., 2004; Pelkmans et al., 2001; Peters et al., 2003; Pol et al., 1999; Tagawa et al., 2005).

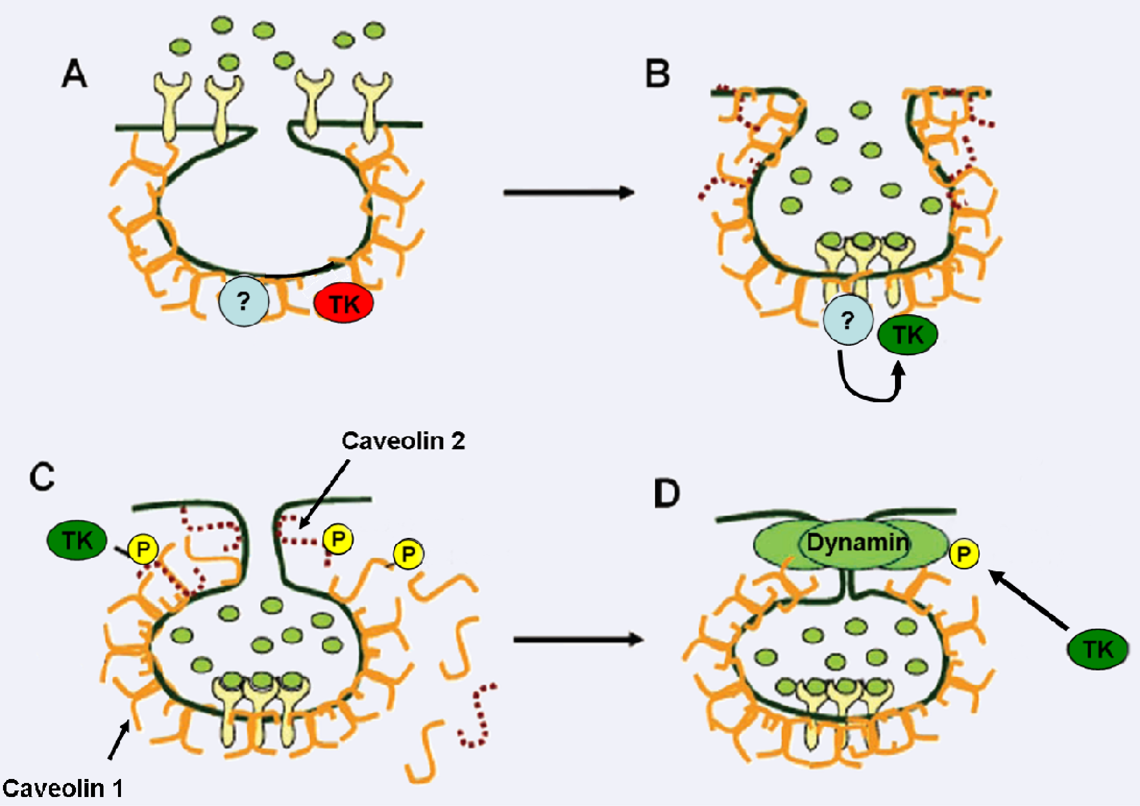

Figure 11. **Caveolae-mediated endocytosis.**
(A) Caveolin 1 acts as a scaffold which stabilizes caveolae at the membrane. (B) Activation of several tyrosine kinases (TK) by an unknown mechanism (?) results in the phosphorylation of caveolin 1, caveolin 2 (C) and dynamin (D) which ultimately leads to the fission of the vesicle. Inactive tyrosine kinases are depicted in red whereas active tyrosine kinases are drawn in green. (Adapted from Sverdlov et al., 2007).

2.4.2 Exomer

The molecular machinery responsible for the formation of post-Golgi carriers directed to the cell surface is still not really understood and the identity of putative coat proteins involved in this process remains elusive. Recently the PtdIns(4)P and Arf1 binding proteins FAPP1 and FAPP2 where shown to be important in this transport pathway (Godi et al., 2004), and FAPP2 was even shown to play a specific role in apical sorting in polarized cells (Vieira et al., 2005). However, both FAPPs were not present on post-Golgi carriers after fission, implicating a role in cargo sorting rather than as new coat proteins.

In 2006 two groups independently proposed a yeast specific coat protein called exomer to be involved in TGN to PM transport (Trautwein et al., 2006; Wang et al., 2006). They investigated the traffic of the yeast chitin synthase III (Chs3p), a protein required to form the chitin ring at the mother-bud junction in growing yeast cells. It translocates from the TGN directly to the cell surface with the help of two peripheral proteins Chs5p and Chs6p (Santos et al., 1997; Ziman et al., 1998). Trautwein et al. (2006) could show that Chs6p belongs to a family with three other members (Bud7p, Ymr237p and Ykr027p) that all bind to Arf1p and Chs5p. Thus the family was named ChAPs (Chs5p-Arf1p-binding Proteins). Based on the findings that the ChAPs are recruited to the TGN in a Chs5p dependent manner, where they can interact with each other, Arf1p-GTP, and Chs3p, it was proposed that the ChAPs together with Chs5p could form a novel coat that was later named exomer. This was confirmed by Wang et al. (2006) who showed that the Chaps/Chs5p complex not only binds Arf-1p but is also recruited to the membrane by it. Sec7p, a GEF involved in secretory traffic within the Golgi complex, was shown to be needed for proper TGN localisation of Chs5p.

A model was proposed where Chs5p is recruited by Arf1p-GTP to the TGN like AP-1 in the clathrin coat. The ChAPs would be recruited by Chs5p forming the exomer complex, which then binds cargo (Chs3p) and transports it to the cell surface. EM studies of exomer-coated liposomes revealed spiky structures whose morphology was quite distinct from other coat complexes. However, no coated buds or small vesicles were detected, suggesting that additional factors are needed to form complete vesicles.

2.5 Vesicle targeting

To ensure that membrane traffic proceeds correctly, it is of crucial importance that newly formed vesicles target and fuse with the correct acceptor membrane in a highly specific manner. Basically, three classes of proteins are involved in this process: SNAREs (soluble NSF attachment protein receptors), tethering factors and the small GTPases of the Rab family.

2.5.1 SNAREs

SNAREs are involved in the final docking process of uncoated vesicles with their target membrane and catalyze membrane fusion (reviewed in Hong, 2005). Most of them are C-terminally anchored transmembrane proteins with a cytosolic N-terminal domain containing a SNARE motif that can participate in coiled coil formation (Bock et al., 2001).

According to the SNARE hypothesis, each vesicle carries a specific v(esicle)-SNARE which binds to a t(arget)-SNARE on the target membrane, resulting in a trans-SNARE complex (Rothman and Warren, 1994). Structural and biochemical studies revealed that such a complex consists of a very stable four-helix bundle, with one α-helix from the monomeric v-SNARE and 3 α-helices from the oligomeric t-SNARE (Fasshauer et al., 1997; Sutton et al., 1998). The free energy that is needed for subsequent membrane fusion is thought to be supplied from the assembly of this helix bundle (Hanson et al., 1997; Lin and Scheller, 1997).

The trans-SNARE complex persists throughout the fusion reaction and becomes a cis-SNARE complex, consisting of v- and t-SNAREs on the same membrane. To disassemble this four-helix bundle and thereby allowing new rounds of fusion, α-SNAP (soluble NSF attachment protein) binds along the edge of the cis-complex (Rice and Brunger, 1999) where it recruits NSF (N-ethylmaleimide-sensitive-factor) which dissociates the complex in an ATP dependent manner (Mayer et al., 1996).

SNAREs were first thought to ensure the specificity of the fusion reaction alone by allowing only certain v-SNAREs to interact with defined t-SNARE complexes (Parlati et al., 2002). However, soon it became clear that there had to be additional proteins to ensure correct vesicle targeting.

2.5.2 Rab proteins

Rab proteins form the largest family within the Ras superfamily of small GTPases. They continuously cycle between the cytosol and membranes and have an important role in vesicle targeting. In the inactive GDP-bound form, Rabs are cytosolic and form a complex with GDI (guanine nucleotide dissociation inhibitor). Upon the action of a GDF (GDI displacement factor), Rabs are recruited to the membrane (Dirac-Svejstrup et al., 1997) and inserted into the membrane via a c-terminal prenyl group. Different Rabs where shown to localize to different compartments. For example Rab11 is found on recycling endosomes, whereas Rab5 and Rab6 are localized on sorting endosomes and Golgi, respectively (Zerial and McBride, 2001).

The membrane bound Rab is activated by a GEF through the exchange of its GDP with GTP (Soldati et al., 1994; Ullrich et al., 1994). Upon activation the Rab can interact with several Rab effectors like tethering factors or motor proteins important to translocate the vesicle along actin filaments or microtubules. In this way Rab proteins speed up the vesicle targeting and fusion process. After vesicle fusion, a GAP terminates the membrane association of the Rab protein by stimulating GTP hydrolysis, leading to its recycling back into the cytosol (Rybin et al., 1996).

2.5.3 Tethering factors

Tethering factors provide additional specificity to vesicle targeting by connecting the donor and acceptor membranes prior to SNARE complex formation. They are thought to mediate the first contact between the vesicle and the target membrane. Almost all tethering proteins can be subdivided into two classes. The first class consists of long putative coiled coil proteins like EEA1 or the golgins that are involved in homotypic fusion of early endosomes or fusion events at the Golgi apparatus, respectively (Barr and Short, 2003; Christoforidis et al., 1999). The second class contains multisubunit complexes like the exocyst, which was shown to be involved in Golgi/endosome-to-plasma membrane transport (TerBush et al., 1996). It was shown that tethering factors can interact not only with Rabs and SNAREs but also with coat proteins (reviewed in Cai et al., 2007). Therefore it was postulated that at least part of the coat of a vesicle is needed until it is recognized by specific tethers on the acceptor membrane (Figure 12). This interaction is thought to be maintained as regions of the vesicle uncoat and the SNAREs are exposed. At the same time other tethers recruited by Rab proteins are thought to promote SNARE pairing and subsequent membrane fusion. However, additional studies will be needed to determine how exactly coats, tethers, SNAREs and Rabs act together to control the fidelity of membrane traffic.

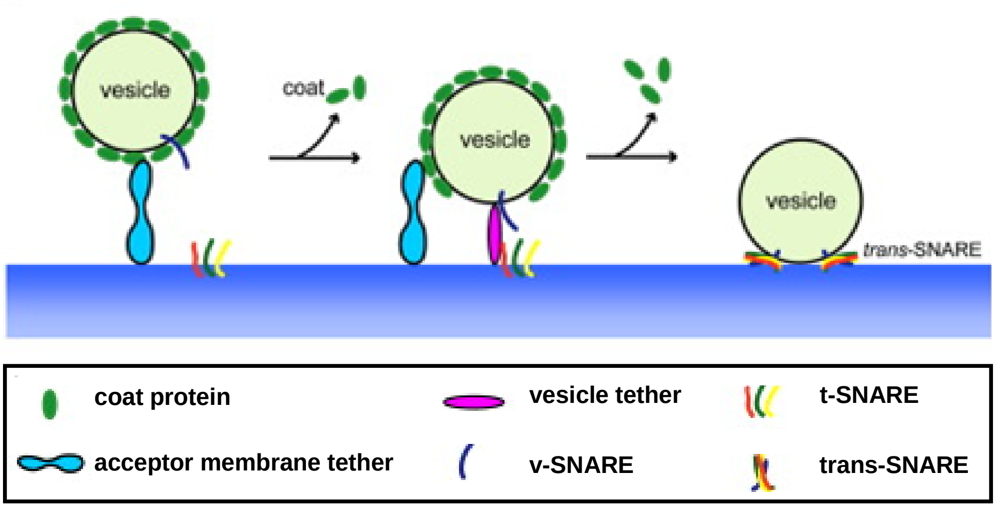

Figure 12.**Vesicle targeting**.
The tether of the acceptor membrane (depicted in blue) recruits the vesicle via a direct interaction with a coat subunit. Upon uncoating of the vesicle the v-SNARES are exposed and vesicle tethers (depicted in pink) promote the formation of a trans-SNARE complex which finally leads to the fusion of the membranes. (Adapted from Cai et al., 2007).

3. The docking factor(s)

Most of what is known about the formation of clathrin coated vesicles comes from studies that examined the recruitment of AP-2 and clathrin at the plasma membrane. Much less is known about factors involved in AP-1 and subsequent clathrin recruitment at the TGN or endosomes. A central question that remains unsolved for example is how exactly AP-1 is recruited to membranes. In vitro studies with purified Golgi membranes or liposomes led to a better understanding of this process.

3.1 A cytosolic factor is required to recruit AP-1

It was shown in vitro that AP-1 recruits to purified Golgi membranes in an Arf1 dependent manner (Stamnes and Rothman, 1993; Traub et al., 1993). However, since under the same conditions COPI is also recruited, there have to be additional factors to define specificity of AP-1 recruitment to the TGN. Trypsin digestion of purified Golgi membranes abolished AP-1 binding, ruling out the possibility that only differences in the lipid composition between Golgi cisternae and TGN are responsible for proper coatomer and AP-1 recruitment, respectively. Thus it was proposed that one or more specific AP-1 docking protein(s) at the TGN act together with Arf1 to ensure specific AP-1 recruitment.

The model of the docking protein was soon refined when Zhu et al. (1998) showed that AP-1 could transiently bind to purified Golgi membranes even after GTP hydrolysis of Arf1. AP-1 that was recruited to Golgi with Arf-GTP was found to be sensitive to Tris extraction and to time- and temperature-dependent release. In the absence of GTP hydrolysis (i.e. with GTPγS or Arf1Q71L), AP-1 was resistant to Tris extraction and remained membrane associated. In two-step experiments, AP-1 could be recruited on ice, following “activation” of Golgi membranes with Arf-GTP at 37°C. However, recruited AP-1 was fully sensitive to Tris extraction, indicating that the activated Arf had already passed the point of Arf1-GTP hydrolysis. Based on these findings it was proposed that activated Arf1 interacts with the docking factor at the TGN leading to a conformational change of the factor which results in the exposure of an AP-1 binding site. Like this, activated Arf1 would generate a high affinity binding site for AP-1. After GTP hydrolysis, reversion of the docking protein to its ground state might take a finite period which would explain AP-1 recruitment after Arf1 hydrolysis.
Hoflack and colleagues proposed that the MPR as the major AP-1 cargo serves as an essential docking site for AP-1 recruitment (Le Borgne and Hoflack, 1997). This theory, however, was

disputed by binding studies with fibroblasts lacking MPR that still showed efficient AP-1 recruitment to Golgi membranes (Zhu et al., 1999a). In fact, it was shown that the docking factor for AP-1 recruitment is not a transmembrane protein. Using protein-free liposomes Zhu et al. (1999b) demonstrated that Arf1 binding to liposomes is not sufficient for recruitment of AP-1 from a CCV coat fraction. However, AP-1 from full cytosol could be recruited suggesting that the docking factor of AP-1 was present in the cytosol. These results could later be confirmed by Crottet et al. (2002) and Meyer et al. (2005). Since purified Golgi membranes, in contrast to liposomes, bound AP-1 directly from CCV coat material, it was proposed that the cytosolic factor is a peripheral membrane protein associated with the Golgi membranes. A similar factor might also exist on immature secretory granules that bind AP-1 in an Arf1-GTP dependent manner, because Tris stripping and the subsequent removal of peripheral membrane proteins abolished AP-1 binding (Dittie et al., 1996).

However, biochemical reconstitution experiments indicate that the minimal set of components necessary for efficient recruitment of AP-1 onto the surface of synthetic liposomes is Arf-GTP, a membrane anchored YXXΦ sorting signal, and phosphoinositides (Crottet et al., 2002). Therefore it seems that at least in vitro a cytosolic factor is not needed as long as cargo signals are present. Yet these cargo signals can not define specificity of AP-1 recruitment, since YXXΦ sorting signals are also recognized by other APs. In vivo it seems likely that AP-1 is first recruited specifically via cytosolic factor(s) to the site of vesicle formation where it therefore can subsequently interact with cargo receptors.

3.2 Candidates for the cytosolic factor(s)

Even though several studies confirmed the existence of a cytosolic docking factor involved in AP-1 recruitment, the identity of the factor was elusive. However, several candidates were proposed:

Mallet and Brodsky discovered two peripheral membrane proteins of 83kD and 52kD that are part of a larger complex (between 260 and 630kD) and bind via the 83kD protein directly to the core domain of AP-1 (Mallet and Brodsky, 1996). This binding was shown to be specific and both proteins cofractionated with TGN membranes. However it was not possible to purify these two proteins in sufficient quantity for mass spectrometry analysis, thus the identity of these two candidates remains unknown. Interestingly, Seaman et al. (1996) also identified a ~80kD protein together with a 60kD and a 75kD protein, using a cross-linking approach to

find new binding partners for AP-1. Also in this case it was not possible to purify the candidates in sufficient quantities for sequence analysis.
The most promising factor candidate so far, the ~30kD protein γ-BAR (γ1-adaptin brefeldin A resistance), was identified by Neubrand and colleagues (Neubrand et al., 2005). The peripheral membrane protein was shown to directly interact with the γ-appendage of AP-1. Immunogold EM studies showed a good colocalization with AP-1 at the TGN and on vesicular profiles. When γ-BAR was overexpressed the fraction of membrane bound AP-1 was not only increased but also resistant to BFA treatment. This protection from BFA was AP-1 specific since AP-3 and GGA3 were not protected. siRNA experiments against γ-BAR on the other hand resulted in AP-1 redistribution to the cytosol. Interestingly γ-BAR localisation was dependent on AP-1. When γ-BAR and AP-1 were monitored over time in cells treated with siRNA against the μ_1 subunit of AP-1, AP-1 was released from membranes followed by γ-BAR, suggesting that γ-BAR can bind membranes independently of AP-1 but fails to do so in the continuous absence of AP-1 from membranes. However, only a final liposome recruitment assay in a minimal system containing only γ-BAR, AP-1, GTPγS and Arf1 will show whether γ-BAR is responsible for the recruitment activity described by Zhu et al. (1999b).

The involvement of a docking factor was not only suggested in AP-1 mediated CCV formation. In vitro experiments with purified plasma membranes of human fibroblast cells revealed the existence of a transmembrane protein that showed high affinity binding to AP-2 and therefore could act as a docking factor at the plasma membrane (Anderson, 1993). However the protein could not be identified. A possible candidate was found several years later. The transmembrane protein synaptotagmin 1 was shown to act as a high affinity receptor for AP-2 (Zhang et al., 1994). Furthermore it was shown that binding of AP-2 to YXXΦ based endocytic signals increased its affinity to synaptotagmin 1. Thus, it would not only help to recruit AP-2 but also couple cargo sorting with coat formation (Haucke and De Camilli, 1999). However synaptotagmin 1 is a neuronal specific protein. The identity of other docking factors involved in general receptor mediated endocytosis remains elusive. Likely candidates would be the ubiquitously expressed isoforms of synaptotagmin although no such role has been reported yet.

3.3 Clathrin coated vesicle formation at the TGN/endosomes

Based on the studies by Kornfeld and colleagues and the findings of our lab, the model shown in Figure 13 could be proposed.

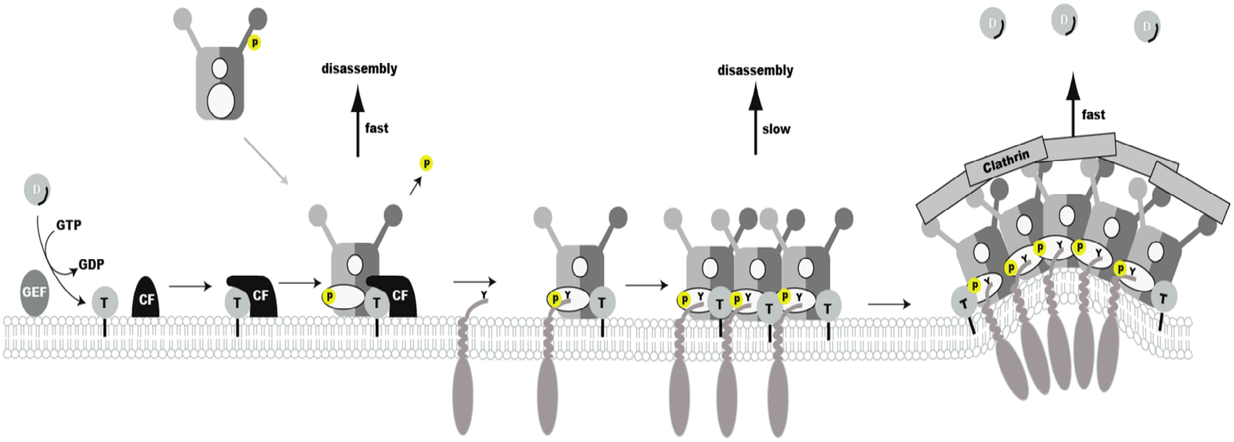

Figure 13. **Formation of CCVs at the TGN/endosomes**.
The grey arrow indicates AP-1 recruitment via Arf-GTP and the cytosolic factor (CF). The lower circle in cytosolic AP-1 represents the “inactive” μ1 subunit prior to its phosphorylation. For simplicity ArfGAP and other accessory proteins involved in AP-1 recruitment are not drawn. For a detailed description see text below.

In a first step, the small GTPase Arf1 is activated by a specific GEF (e.g., BIG1 or BIG2) resulting in the exposure of its myristoyl tail and an N-terminal amphiphatic α-helix which allows membrane insertion. Activated Arf1 then interacts with a membrane associated docking factor resulting in a conformational change of the factor which thereby reveals an AP-1 binding site. Through interactions with Arf1, the docking protein and PtdIns(4)P, AP-1 is recruited to the membrane. The resulting complex then interacts with ArfGAP1 and stimulates its GAP activity which leads to a rapid dissociation of the complex in the absence of cargo, preventing the formation of empty vesicles (Meyer et al., 2005). However if cargo is present, AP-1 binds to it, extending the time it remains associated with the membrane.

Cargo binding of AP-1 was shown to be a highly regulated process: In the cytosol, AP-1 is in an inactive conformation, since it is selectively phosphorylated on its β-hinge, which impairs its binding to clathrin (Wilde and Brodsky, 1996), and its sorting signal binding site in the μ1 subunit is hidden in the core. Upon membrane recruitment, β1 is dephosphorylated by a still unknown membrane associated phosphatase, allowing its interaction with clathrin. At the same time μ1 is phosphorylated - most probably by the γ-appendage associated GAK - which leads to the exposure of its ligand binding site and thereby enhances its interaction with cargo. Cargo binding was shown to induce a conformational change in the core of AP-1 that enhances its association with Arf1 (Lee et al., 2008). Additionally, AP-1 binding to tyrosine signals was proposed to reduce AP-1 dependent stimulation of GAP activity analogous to coatomer (Goldberg, 1999; Meyer et al., 2005). Thus, cargo sorting signals play an active role in promoting their own sorting by ensuring the stable association of AP-1 through both a stronger association with activated Arf1 and a reduction of the ArfGAP1 activity. This provides AP-1 sufficient time to oligomerize and finally recruit and assemble the clathrin coat. It has been shown that positive membrane curvature strongly stimulates ArfGAP1 activity (Bigay et al., 2003); therefore it is thought that membrane deformation by clathrin and accessory proteins enhances GTP hydrolysis and release of Arf1-GDP.

After vesicle budding, clathrin disassembly was shown to be catalyzed by Hsc70 and its cofactor auxilin or GAK (Umeda et al., 2000; Ungewickell et al., 1995). Additionally β1 phosphorylation is thought to contribute to this process. The AP-1 layer, on the other hand, is released through the action of Hsc70 and PP2A which dephosphorylates μ1 resulting in a decreased avidity of AP-1 for sorting signals and its subsequent dissociation from the membrane (Ghosh and Kornfeld, 2003a).

Aim of this thesis

We have observed that the minimal requirements for AP-1 recruitment to liposomes are specific phosphoinositides, activated Arf1, and either sorting signals or an unknown cytosolic factor. These findings are in agreement with Kornfeld and colleagues who proposed that a cytosolic docking factor is required to specifically recruit AP-1 to the TGN/endosomes. However, the identity of this factor remained elusive.

The goal of this work was to identify this cytosolic docking factor. To address this question, calf brain cytosol was separated on several chromatography columns and the different fractions were tested for the presence of the factor using a liposome recruitment assay. Mass spectrometry analysis together with immunodepletion experiments finally allowed the identification of the factor. Furthermore our studies also addressed the minimal requirements for membrane recruitment of the factor which resulted in a novel model of AP-1 recruitment at the TGN/endosomes.

Materials and methods

Materials

Reagents – Guanylyl imidodiphosphate (GMP-PNP) and dimethylpimelidate were from Fluka. ECL reagent was from Millipore (Billerica, MA). MMCC-DOPE (N-((4-maleimidylmethyl)cyclohexane-1-carbonyl)-1,2-dioleolyl-sn-glycero-3-phosphoethanolamine was from Avanti Polar Lipids (Alabaster, AL). Soybean phospholipids containing 20% PC (azolectin, P-5638), Coomassie brilliant blue R-250, trypsin from bovine pancreas, trypsin inhibitor, benzamidine, pepstatin, leupeptin, antipain and chymostatin were all purchased from Sigma (Buchs, Switzerland). The Bradford protein assay and the Precision Plus (All Blue Standards) molecular marker were from Bio-Rad (Hercules, CA). Phenylmethylsulfonyl fluoride (PMSF), bactotryptone and yeast extracts were from Applichem (Darmstadt, Germany). Peptides were purchased at >70% purity from NeoMPS (Strasbourg, France). Peptide sequences were CRKRSHAGYQTI-COOH (Lamp-1Y), CRKRSHAGAQTI-COOH (Lamp-1A), and DLLGDINLTGAPAAAPA (competing peptide for 100/3).

Antibodies **–** Mouse anti-amphiphysin I (8) from Santa Cruz Biotechnology, mouse anti-amphiphysin II (99D) from Sigma, and mouse anti-γ-adaptin (100/3) produced in hybridoma cells obtained from ATCC, were used for immunoprecipitation (IP) experiments.

For immunoblotting experiments the following antibodies were used at the indicated dilutions: mouse anti-amphiphysin I (13) (1:4000); goat anti-amphiphysin II (N-19) (1:4000); mouse anti-endophilin I-III (G-8) (1:4000), all from Santa Cruz Biotechnology; mouse anti-γ-adaptin (100/3) (1:4000); mouse anti-Arf1 (1D9) (1:5000) from Alexis Biochemicals; mouse anti-β1/2-adaptin (100/1) (1:4000) from Sigma; rabbit anti-μ1A antiserum raised against the synthetic peptide EAEDKEGKPPISV (1:1000); mouse anti-α-adaptin (100/2) (1:4000) from Sigma.

As secondary antibodies, horseradish peroxidase-coupled goat anti-rabbit (1:10’000), goat anti-mouse (1:10’000), and rabbit anti-goat (1:4000) IgG antibodies (all from Sigma) were used as appropriate at the indicated dilution.

***Columns* –** The following columns were used in this thesis:

Name	**Dimensions**	**Source**
Superdex 75 (HighLoad 26/60 prep grade)	2.6 x 60 cm	GE Healthcare
Superdex 200 (HighLoad 26/60 prep grade)	1.6 x 60 cm	GE Healthcare
CHT5-I hydroxyapatite	1.0 x 6.4 cm	Bio-Rad Laboratories
Mono Q (10/100 GL)	1.0 x 10 cm	GE Healthcare
Mono S (10/100 GL)	1.0 x 10 cm	GE Healthcare
PD-10 desalting (Sephadex G-25)	8.3 mL	GE Healthcare
Superose 6 prep grade, packed into an HR 16/60	1.6 x 60 cm	GE Healthcare
Hydrophobic interaction column (HiPrep 16/10 Phenyl FF (high sub))	1.6 x 10 cm	GE Healthcare
Econo-Column	0.7 x 4 cm	Bio-Rad Laboratories
Econo-Column	1.0 x 10 cm	Bio-Rad Laboratories

Protein purification and liposome preparation

All procedures were performed at 4°C unless expressly stated.

Isolation of cytosol and clathrin coated vesicles (CCVs) – The preparation of cytosol and the isolation of CCVs has been described in detail in Suri et al. (2008). In brief, six calf brains obtained at the local slaughterhouse were cleaned from fat, brain stem and meningaes, mixed in a Waring blender (Waring laboratory, Torrington, CT) with half their volume of buffer A (0.1 M MES-NaOH, pH 6.6, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT) and supplemented with 0.5 mM PMSF. The homogenate was centrifuged at 8000 x g for 30 min and the supernatant was subjected to a high speed centrifugation of 180’000 x g for 80 min to pellet the membranes. The resulting high-speed supernatant, i.e. the cytosol, was collected, aliquoted and shock frozen in liquid nitrogen. The cytosol was stored at -80°C for several weeks. Protein concentration was typically ~ 28µg/µL as determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as standard.

To purify CCVs the pellet of the high speed centrifugation were dissolved in ~ 8 mL buffer A and homogenized in a medium Dounce homogenizer (Bellco Biotechnology, Vineland, NJ). The homogenate was mixed with an equal volume of buffer B (0.1 M MES-NaOH, pH 7, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 12.5% (w/v) Ficoll400, 12.5% (w/v) sucrose) re-Dounce homogenized and centrifuged at 60’000 x g for 40min. The supernatant was collected, diluted with 3 volumes of buffer A and centrifuged at 180’000 x g for 80min to pellet the CCVs. The pellets were resuspended in an equal volume of buffer A, homogenized in a small Dounce homogenizer (Bellco Biotechnology, Vineland, NJ) and centrifuged in a microfuge for 12min to remove aggregated material. The supernatant containing the CCVs was collected, shock-frozen in liquid nitrogen and stored at -80°C.

Preparation of mixed adaptors – Mixed adaptors were essentially produced as described in Suri et al. (2008). To release coat proteins, CCVs were homogenized in a small Dounce homogenizer with one volume of stripping buffer (1.5 M Tris-HCL, pH 7.0, 6 mM EDTA, 0.6 mM DTT) and supplemented with 0.5 mM PMSF and 1 x Protease inhibitor cocktail (PIC) from a 500x stock (5 mg/mL benzamidine, 1 mg/mL pepstatin A, leupeptin, antipain, and chymostatin). The mixture was incubated on ice over night before membranes were pelleted for 35 min at 100’000 x g. The supernatant was loaded in 2 mL portions on a 1.6 x 60 cm Superose 6 column that was preequilibrated in Superose buffer (0.5 M Tris-HCl, pH 7.0, 2 mM EDTA, 0.2 mM DTT) and 1 mL fractions were collected. AP-1 containing fractions were pooled (typically fractions 62-70) and supplemented with 1x PIC and 0.5 mM PMSF. Protein concentration was determined to be 0.5 µg/µL using the Bradford assay.

Coupling of anti-γ-adaptin (100/3) to protein A-sepharose beads – 1.5 mg anti-γ-adaptin (100/3) were diluted in 50 mM Tris-HCl (pH 9) before 1.2 mL packed proteinA sepharose beads (Invitrogen, Carlsbad, CA) were added. The mixture was incubated at room temperature for 1 h end-over-end before the beads were washed twice with 50 mM Na-borate (pH 9). The beads were resuspended in 20 mL of 0.2 M Na-borate (pH 9) and 104 mg of dimethylpimelidate was added to covalently link the antibodies to the beads. After a 30 min incubation at room temperature on an end-over-end shaker, the beads were washed once with 0.2 M ethanolamine (pH 8.0) and incubated with 20 mL of 0.2 M ethanolamine (pH 8.0) on an end-over-end rotator for 2 h. Thereafter beads were washed and resuspended 1:1 in PBS. The amount of coupled antibody was determined by Coomassie.

Isolation of AP-1 from cytosol – 10 mL of cytosol were supplemented with 0.5 mM PMSF and 1x PIC and centrifuged for 1 h at 10’000 x g to remove insoluble aggregates. Thereafter the cytosol was incubated with anti-γ-adaptin (100/3) coupled to 1 mL of protein A-Sepharose on an end-over shaker over night at 4°C. The cytosol together with the beads was loaded on a Econo-Column (1.0 x 10 cm) and washed with 20 mL of buffer A without DTT (see above) at 4°C. Bound AP-1 was released by incubating the beads 3 times with a 100x excess of a competing peptide (DLLGDINLTGAPAAAPA) dissolved in 1 mL buffer A for 30 min at 37°C (Stuart Kornfeld, personnel communication). The released AP-1 was supplemented with 0.5 mM PMSF and 1x PIC and stored at 4°C for up to 3 days.

Isolation of AP-1 from CCV coats – AP-1 was basically isolated from CCV coats like described in Meyer et al. (2005). In short, CCVs were stripped over night and loaded on a CHT-I hydroxyapatite column (1.0 x 6.4 cm) that was preequilibrated with hydroxyapatite buffer (0.5 M Tris-HCl pH 7, 2 mM K-phosphate, 0.2 mM DTT). AP-1 was eluted stepwise with 50 mM and 100 mM phosphate. Fractions containing AP-1 were pooled and the buffer was changed to Mono Q buffer (20 mM ethanolamine, pH 8.9, 2 mM EDTA, 1 mM DTT) with the help of an Amicon Ultra 15 with a cut-off of 50 kD (Milipore). Thereafter the sample was loaded on a Mono Q column (1.0 x 10 cm) and purified AP-1 was eluted with an increasing salt gradient from 0-0.5 M NaCl. AP-1 containing fractions were pooled, supplemented with 0.5 mM PMSF and 1x PIC and stored at 4°C for up to 1 week.

Preparation of myristoylated Arf1-His$_6$ – Myristoylated Arf1 was purified as a His-tagged version from bacteria. Plasmids encoding bovine Arf1-His$_6$ (pET Arf1-His$_6$) and yeast N-myristoyltransferase (pBB131; (Duronio et al., 1990)) were cotransformed into *Escherichia coli* BL21 (DE3). The bacterial suspension was incubated with 1 mL Luria broth (LB; 10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C for 1 h before the addition of 25 mL LB with 100 μg/mL each ampicillin and kanamycin. The mixture was incubated overnight with shaking to select for transformants carrying both plasmids. The overnight culture was used to inoculate 1L LB with 100 μg/mL each of ampicillin and kanamycin. The culture was grown at 37°C until OD_{600} of 0.4 was reached. Arf1 and NMT expression was induced by the addition of 1 mM IPTG. Simultaneously, 0.5% (w/v) Brij 58 and 500 μM myristic acid were added and the culture was incubated at 30°C for 4 h with shaking. Cells were harvested by centrifugation for 10 min at 2700 x g and washed in water before they were recentrifuged at 4300 x g for 10min. The pellets were stored at -80°C. Cells were lysed on ice by resuspending them in 20 mL of STE buffer (50 mM Tris, pH 8.0, 40 mM EDTA, 25% (w/v) sucrose) supplemented with 0.5 mM PMSF and 1x PIC. Lysozyme was added to a final concentration of 1 mg/mL and the mixture was incubated for 15 min at room temperature. After sonication (on ice, 8 x 15sec, 1min recovery, 25% power, constant duty,) in a Branson Sonifier 250, 8 mL of TX-100 buffer (0.2% TX-100, 50 mM Tris, pH 8.0, 100 mM $MgCl_2$) was added and the mixture was centrifuged for 15 min at 27’000 x g. The supernatant was recentrifuged for 30 min at 48’000 x g. Thereafter the supernatant was supplemented with 100 mg DNAse and rotated 1h at 4°C. After that 10 mM imidazole was added and the mix was incubated with 1 mL Ni-NTA beads preequilibrated in 50 mM Tris-HCL, pH 7.4 for 2 h. Thereafter beads were washed 3 times with 10 mL of washing buffer (50 mM Tris pH 7.4, 1 mM EDTA 50 mM NaCl 1 mM DTT) before everything was loaded on an Econo-Column (1.0 x 10 cm). Five 1 mL fractions were eluted and immediately after elution supplemented with 50 μL Tris-HCL, pH 7.4, and 1 mM DTT. Fractions were analyzed by SDS-PAGE and Coomassie staining before Arf1 containing fractions were pooled and loaded on a Superdex 75 column (2.6 x 60 cm). 3 mL fractions were eluted and analyzed by SDS-PAGE and Coomassie. Arf1-His$_6$ containing fractions were pooled and concentrated to 1 mg/mL by ultrafiltration (Amicon Ultra 15 with a cut-off of 10 kD, Milipore). The purified protein was aliquoted, shock frozen in liquid nitrogen and stored at -80°C.

Preparation of peptidoliposomes – Peptidoliposomes made of 97.5% soybean phospholipids were essentially prepared as described in Suri et al. (2008). In short, 5 µmole of soybean lipids (3.8 mg) were dissolved in 1 mL chloroform/methanol and mixed with NBD-PE (1 mole %). If a signal peptide was added, the mixture was supplemented with 125 nmoles MMCC-DOPE (2.5 mole %). The organic solvent was evaporated under a stream of nitrogen before dichloromethane was added and evaporated twice. Dried lipids were resuspended in 1 mL liposome buffer (10 mM HEPES-NaOH, pH 6.5, 100 mM NaCl, 0.5 mM EDTA) by five cycles of vortexing, shock-freezing in liquid nitrogen, and thawing. Thereafter, lipids were extruded 15 times through a 400nm nucleopore polycarbonate membrane (Whatman, Brentford, UK) using a homemade hand-driven extruder.

If a signal peptide was added, the liposomes were mixed immediately after extrusion with 100 µL 4 mg/mL peptide (i.e., about a fourfold excess over the coupling lipid, assuming half of it is exposed), incubated for 1 h at room temperature, supplemented with 0.02% NaN_3 and then stored at 4°C for up to 3 days. When there was no peptide to be added the liposomes were stored at 4°C immediately after extrusion. We found it unnecessary to remove free peptides (e.g. by gel filtration or dialysis) from the liposomes before the liposome recruitment assay (negligible inhibition of adaptor binding to immobilized peptides had also been observed in surface plasmon resonance assays; (Heilker et al., 1996))

Biochemical methods

All procedures were performed at 4°C unless expressly stated

Liposome recruitment assay – The liposomal recruitment assay was basically done as described in Suri et al. (Suri et al., 2008) In brief, 100 µL of liposomes (0.5 µmol lipid) with or without coupled peptides were incubated for 30 min at 37°C with 2 µg Arf1-His, 0.2 mM GMP-PNP, 10 µg of mixed adaptors or 4 µg AP-1 , 4.8 mg/mL Bovine serum albumin (BSA) to block unspecific binding, and the indicated amounts of a protein fraction filled up to 330 µL with assay buffer (0.1 M MES-NaOH, pH 6.6, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT). The final reaction of 480 µL was mixed with 480 µL of 60% (w/v) sucrose in assay buffer overlaid with 3 mL of 30% sucrose in assay buffer and with 0.18 mL of buffer in a 4 mL centrifuge tube, and centrifuged at 300’000 x g for 1.5 h at 4°C. 1 mL fractions were collected from the top and precipitated with 17% (w/v) trichloroacetic acid after 5 µg of BSA was added as a carrier. Acetone washed pellets were boiled in SDS-sample buffer (4% SDS, 0.16 M Tris (pH 6.8), 8.7% glycerol, 10 mM DTT, 0.05% Bromo-phenolblue, 0.4 M β-mercaptoethanol) separated by 10-12.5% PAGE and transferred to a polyvinylidene fluoride membrane (Milipore) for 1 h before the membrane was incubated in blocking buffer (PBS with 0.1% Tween-20 and 5% nonfat dry milk) for 30 min. Primary and secondary antibodies were incubated with the membrane for 60 and 30 min, respectively, and bound antibody was detected by ECL.

Sedimentation assay – The sedimentation assay was performed as described in Meyer et al. (2005) without the addition of Triton X-100. In short, 288 μg of a cytosolic factor-containing Mono Q fraction were loaded on top of a 4.32 mL 10-25% (w/v) sucrose gradient in assay buffer that was prepared with a Gradient Master (BioComp Instrument). The gradient was centrifuged at 90’000 x g for 48 h at 4°C and 10 0.5 mL fractions were collected from the top and examined by immunoblotting. For a molecular weight standard, the experiment was repeated with 70 μg of mixed APs.

Immunodepletion of amphiphysin 1, amphiphysin 2 and AP-1 – To deplete a 40-μg Mono Q fraction of Amphiphysin 1 or 2, 40 μL of proteinA sepharose beads (Invitrogen) that were washed twice in phosphate buffer saline (PBS; 12.5 mM Na_2HPO_4, pH 7.6, 125 mM NaCl), were incubated with 1 mL of PBS containing 40 μg of anti-amphiphysin I (8) or anti-amphiphysin II (99D), respectively, and supplemented with 50 μL of 1 M Tris-HCl pH 8. The mixture was incubated overnight at 4°C on an end-over-end shaker. Thereafter, the beads were washed twice in PBS and incubated with 40 μg of a Mono Q fraction for 2 h at 4°C on an end-over-end shaker. Beads were separated from the immunodepleted supernatant by centrifugation and examined by immunoblotting. The supernatant was examined by immunoblotting and used in a liposomal recruitment assay.

The immunodepletion of AP-1 was performed similarly, using 20 μg anti-γ-adaptin (100/3) to deplete 3.5 mg cytosol.

Proteinase K digestion – 40 μL (1.12 mg) of cytosol was mixed with an equal volume of assay buffer and incubated with 1-2 units of preswollen proteinase K immobilized on agarose beads (Sigma) for 1 h at 37°C on an end-over-end shaker. Thereafter, proteinase K was separated from the mixture by filtration on an Econo-Column (0.7 x 4 cm) leaving the beads with the coupled proteinase K behind. The digested sample was supplemented with 0.5 mM PMSF and 1x PIC.

***Limited trypsin digestion* –** 8 µg AP-1 isolated from cytosol was incubated with 2 µg trypsin for 30 min at room temperature in a total reaction volume of 40 µL. The reaction was stopped with the addition of 10 µg trypsin inhibitor and 10 mM PMSF, and incubated 15 min on ice. Half of the sample was examined by immunoblotting, whereas the other half was used in a liposome recruitment assay.

Delipidation – To remove any remaining lipids, 1,1,2-trichloro-trifluoroethane was mixed with calf brain cytosol in a 3:2 ration and incubated for 1.5 h on an end-over-end shaker at room temperature. Thereafter the mixture was centrifuged in a microfuge for 10 min at full speed before the lipid-free top layer was collected.

Gel filtration – 30 mg of cytosol was supplemented with 0.5 mM PMSF and 1x PIC before it was centrifuged for 20 min at 245’000 x g to remove insoluble aggregates. The supernatant (2ml) was loaded on a Superdex 200 sizing column (1.6 x 60 cm). 1 mL fractions were collected and analyzed by immunoblotting. To examine the presence of the cytosolic factor 330 µL of the samples were used in the liposome recruitment assay.

Purification of the cytosolic factor

Unless expressly stated, all procedures were performed at 4°C. All columns were connected to a fast protein liquid chromatography (FPLC) system from GE Healthcare (Piscataway, NJ, formerly Pharmacia)

Ammonium sulfate precipitation – 20 mL of cytosol were supplemented with 1x PIC and 0.5 mM PMSF and mixed with 100% saturated (sat.) ammonium sulfate solution to the indicated final concentration (typically 30% (sat.)). This mixture was incubated for 1 h under constant stirring at 4°C before it was centrifuged for 30 min at 10’000 x g. The supernatant was removed and the pellet dissolved in 5 mL assay buffer and homogenized with the help of a small Dounce homogenizer. Thereafter the homogenate was desalted over two PD10 columns.

Hydrophobic interaction chromatography (HIC) – After a 30% (sat.) ammonium sulfate precipitation of 20 mL cytosol and desalting over a PD10 column, the solute was supplemented with 5 M NaCl and dissolved in assay buffer to a final concentration of 2 M NaCl. The sample was centrifuged for 10 min at 10’000 x g to remove undissolved precipitated proteins before it was loaded on a 1.6 x 10 cm hydrophobic interaction column. The sample was eluted with a decreasing salt concentration reaching from 2 to 0 M NaCl in assay buffer. Cytosolic factor-containing fractions (typically fractions 181-200) were collected and concentrated to a final volume of 2.5 mL by ultrafiltration (Amicon Ultra 15 with a cut-off of 50 kD, Milipore).

Mono Q anion chromatography – The eluate of the hydrophobic interaction column, containing the cytosolic factor was loaded on a 1.0 x 10 cm Mono Q column that was preequilibrated in assay buffer and eluted in an increasing salt gradient from 0-1 M NaCl. Cytosolic factor-containing fractions (typically fractions 29-36) were concentrated to a final volume of 2.5 mL and the buffer was changed to assay buffer by ultrafiltration (Amicon Ultra 15 with a cut-off of 50 kD, Milipore). This sample referred to as “Mono Q”-sample was the starting material for different experiments.

Mono S cation chromatography – To purify the factor even further, the Mono Q fraction containing the cytosolic factor was loaded on a 1.0 x 10 cm Mono S column that was preequilibrated in assay buffer. Proteins were eluted with an increasing salt gradient from 0-1M NaCl. The collected fractions were concentrated to a final volume of 2.5 mL and the buffer was changed to assay buffer by ultrafiltration (Amicon Ultra 15 with a cut-off of 50 kD, Milipore). Samples were either examined by Coomassie and subsequent mass spectrometry or subjected to the liposome recruitment assay. Typically the cytosolic factor eluted in fractions 29-36.

Floating – As a last purification step to separate the factor from non floated proteins, 80 μL of fraction 1 of the liposome recruitment assay was analyzed by silver staining (Morrissey, 1981) and subsequent mass spectrometry.

Hydroxyapatite chromatography – The buffer of a cytosolic factor-containing fraction after a 30% (sat.) ammonium sulfate precipitation and a Mono Q column was changed to hydroxyapatite buffer (0.5 M Tris-HCl pH 7, 2 mM K-phosphate, 0.2 mM DTT) by a PD-10 column. The sample was loaded on a 1.0 x 6.4 cm CHT-I hydroxyapatite column and the proteins were released with an increasing amount of phosphate from 0-500 mM K-phosphate. The eluted fractions were concentrated to 1 mL and the buffer was changed to assay buffer by ultrafiltration (Amicon Ultra 15 with a cut-off of 50 kD, Milipore). Samples were then either examined via Coomassie or subjected to the liposome recruitment assay. To examine the membrane bound material 80 µL of fraction 1 of the liposome recruitment assay was analyzed by silver staining and subsequent mass spectrometry. The cytosolic factor typically eluted in fractions 13-24.

Mass spectrometry – Proteins were cut from Coomassie or silver stained acrylamide gels. After in-gel digestion with trypsin, peptides were separated by capillary liquid chromatography using a 300SB C-18 column (Agilent Technologies, Basel, Switzerland) and analyzed on an Orbitrap FT hybrid instrument (Thermo Finnigan, San Jose, CA, USA). Protein identification was done using the SEQUEST software (Eng et al., 1994) against the NCBI non-redundant database.

Results-Part I

Recruitment of coat proteins to peptidoliposomes

Recruitment of Coat Proteins to Peptidoliposomes

Gregor Suri, Martin Spiess, and Pascal Crottet

Summary

Intracellular transport between compartments within the cell is generally mediated by membrane vesicles. Their formation is initiated by activation of small GTPases that then recruit cytosolic proteins to the membrane surface to form a coat, interact with cargo proteins, and deform the lipid bilayer. Liposomes proved to be a useful tool to study the molecular mechanisms of these processes *in vitro*. To analyze the involvement of membrane proteins, the cytosolically exposed sequences may be coupled chemically to reactive lipids in the membrane. Here we describe the use of such peptidoliposomes presenting lipid-coupled cytosolic tails of cargo proteins for the *in vitro* analysis of the membrane recruitment of AP-1 adaptors in the process of forming AP-1/clathrin coats. AP-1 recruitment is mediated by the GTPase Arf1, requires specific lipids, and cargo signals. Interaction with cargo induces AP-1 oligomerization already in the absence of clathrin.

Key words: Arf1; clathrin adaptor protein; coat protein; liposome; membrane traffic; peptidoliposome; protein sorting.

1. Introduction

Liposomes are widely used to study molecular processes at membrane surfaces *in vitro*. They have been particularly useful in the area of membrane trafficking to reconstitute the assembly of cytosolic coat proteins at the lipid bilayer and the formation of coated vesicles from purified components. Lipid and protein compositions, the order of addition of individual components and the conditions (temperature, nucleotides, etc.) can be easily manipulated and help to define the minimal machinery of coat assembly and their molecular mechanisms.

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The three best characterized coats are coat protein (COP) I mediating intra-Golgi and Golgi to endoplasmic reticulum (ER) transport, COPII for vesicles derived from the ER, and clathrin with various associated adaptor proteins (APs) for pathways between the plasma membrane, endosomes, and the *trans*-Golgi network *(1)*. In all systems, coat recruitment is initiated by a small GTPase that is activated at the membrane by nucleotide exchange from GDP to GTP. The generation of COPI vesicles *in vitro* requires the heteroheptameric coatomer complex and ADP-ribosylation factor 1 (Arf1; *2,3)*. COPII consists of two components: Sec23/24 is first targeted by Sar1·GTP to the membrane to recruit the second layer of Sec13/31 *(4)*. Clathrin coats are similarly composed of two layers, heterotetrameric adaptor complexes and clathrin *(5)*.

In all systems, specific lipid compositions are required for coat recruitment, acidic phospholipids for COPI *(3)*, and phosphoinositides for COPII *(4)* and clathrin adaptors *(6)*. In addition, cargo proteins influence coat formation. For example, presentation of cytoplasmic cargo sequences on the membrane surface is important for efficient recruitment of COPI and AP-1 clathrin adaptors *(2,6)* and enhances the stability of membrane-bound Sec23/24 of COPII *(7)*. Different approaches have been used to study the role of cargo proteins. Cargo SNAREs, such as Bet1p and Sec22p, were purified and reconstituted into liposomal membranes by detergent dialysis *(7)*. Alternatively, cytoplasmic sequences of cargo proteins were coupled to liposomes via modified lipids. This can be accomplished by preparing fusion proteins with glutathione-*S*-transferase (GST) that bind tightly to a glutathione-phosphatidylethanolamine conjugate incorporated into the liposomal membrane *(8)* or by covalently coupling synthetic peptides to a maleimide lipid via the sulfhydryl group of a cysteine side chain *(2,6,9)*.

Here we describe the methods to study the *in vitro* recruitment of AP-1 adaptors to peptidoliposome by a floatation assay and to analyze the oligomeric state of recruited AP-1 by sedimentation into a density gradient. We provide protocols to produce adaptor complexes from calf brain clathrin-coated vesicles (CCVs), myristoylated Arf1 from a bacterial expression system, and peptidoliposomes using maleimide lipids, as well as protocols of the floatation and the density gradient sedimentation assays.

2. Materials

Dithiothreitol (DTT) is added just before use from a 1 *M* frozen stock. All the buffers for fast protein liquid chromatography (FPLC) are filtered through 0.2-μm nitrocellulose filters (Millipore) and degassed.

2.1. Isolation of Cytosol and CCVs

1. Two calf brains fresh from the slaughterhouse (*see* **Note 1**).
2. CB-6 Three-speed extra-large capacity blender from Waring Laboratory or equivalent.

3. Loose-fitting dounce homogenizer (Bellco Biotechnology) small (7 mL) and medium (40 mL) size.
4. Phosphate-buffered saline (PBS): 12.5 m*M* sodium phosphate buffer, pH 7.6, 125 m*M* NaCl.
5. Phenylmethylsulfonyl fluoride (PMSF) at 0.5 *M* in dimethyl sulfoxide.
6. Buffer A: 0.1 *M* MES-NaOH, pH 6.6, 0.5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.2 m*M* DTT.
7. Buffer B: 0.1 *M* MES-NaOH, pH 7.0, 0.5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.2 m*M* DTT, 12.5% (w/v) Ficoll 400, 12.5% (w/v) sucrose (*see* **Note 2**).

2.2. Preparation of Mixed Adaptors

1. Fast protein liquid chromatography (FPLC) system (e.g., äkta FPLC system from GE Healthcare).
2. Superose 6 prep grade is packed into an HR 16/60 column (1.6 × 60 cm; both from GE Healthcare).
3. Monoclonal antibodies for immunoblotting (all used at 1:1,000 dilution): 100/3 against γ-adaptin of AP-1 (~90 kD), 100/2 against α-adaptin of AP-2 (doublet at ~100 kD; both from Sigma), and anti-clathrin heavy chain (from BD Biosciences).
4. Stripping buffer (3X): 1.5 *M* Tris-HCl, pH 7.0, 6 m*M* ethylenediamine-tetraacetic acid (EDTA), 0.6 m*M* DTT. This buffer can be stored at –20°C in 15-mL Falcon tubes. Falcon tubes do not resist shock-freezing.
5. Superose running buffer: 0.5 *M* Tris-HCl, pH 7.0, 2 m*M* EDTA, 0.2 m*M* DTT.
6. Protease inhibitor cocktail (500X): 5 mg/mL benzamidine, 1 mg/mL pepstatin A, 1 mg/mL leupeptin, 1 mg/mL antipain, 1 mg/mL chymostatin (all from Sigma); dissolved in 40% dimethyl sulfoxide (DMSO)/60% ethanol and stored at –20°C.

2.3. Preparation of Myristoylated Arf1

1. Plasmids pET-mArf1* encoding bovine Arf1 with codons 3 to 7 replaced by those from yeast Arf2p ***(10)*** and pBB131 encoding yeast myristoyl-CoA:protein *N*-myristoyltransferase (NMT; ***11)*** were provided by Drs. Stuart Kornfeld and Jeffrey Gordon (Washington University, St. Louis, MO), respectively.
2. Competent *Escherichia coli* BL21(DE3) from Stratagene.
3. Monoclonal antibody for immunoblotting 1D9 against Arf (used at 1:5,000 dilution; from Alexis).
4. Diethylaminoethyl (DEAE) Sephacel (from GE Healthcare) in an Econo-Column (2.5 × 10 cm) from Bio-Rad.
5. Superdex 75 column (HighLoad 26/60 prep grade; 2.6 × 60 cm; from GE Healthcare).
6. Amicon Ultra–15 centrifugal filter devices with a 10 kD cut-off from Millipore.
7. Luria broth (LB): 10 g/L bactotryptone (Applichem), 5 g/L yeast extract (Applichem), 10 g/L NaCl.
8. Ampicillin (1,000X): 100 mg/mL in water; store frozen.
9. Kanamycin (500X): 50 mg/mL in water; store frozen.

10. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) is dissolved at 1 *M* in water and stored frozen.
11. Brij 58 is prepared at 10% (w/v) in water and autoclaved.
12. Myristic acid is dissolved at 0.5 *M* in ethanol and stored frozen.
13. DEAE buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$, 10 μ*M* GDP, 0.02% (w/v) NaN_3, 1 m*M* DTT.
14. Superdex running buffer: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* $MgCl_2$ 10 μ*M* GDP, 0.02% (w/v) NaN_3, 10% (w/v) sucrose, 1 m*M* DTT.

2.4. Preparation of Peptidoliposomes

1. Synthetic peptides LY (amino acid sequence CRKRSHAGYQTI) and LA (CRKRSHAGAQTI) correspond to the cytoplasmic domain of Lamp1 (lysosome-associated membrane protein-1) and a transport-deficient mutant with the critical tyrosine mutated to alanine, respectively. Peptides were purchased at more than 70% purity from, e.g., NeoMPS. The N-terminal cysteines were added to the sequences for coupling.
2. Glass tubes resistant to liquid nitrogen, e.g., 12-cm test tubes NS 14.5/23 for vacuum from Glas Keller.
3. Nucleopore polycarbonate membrane with 400-nm pore size and prefilters (drain disc 10 mm PE) from Whatman.
4. Mini-Extruder from Avanti Polar Lipids. For description *see* http://www.avantilipids.com/extruder.html.
5. Egg L-α-phosphatidylcholine (PC) from Avanti Polar Lipids.
6. Soybean L-α-phosphatidylcholine, also called azolectin (Sigma). This is a mixture of phospholipids, containing only 20% PC, however.
7. MMCC-DOPE (*N*-[{4-maleimidylmethyl}cyclohexane-1-carbonyl]-1,2-dioleo lyl-sn-glycero-3-phosphoethanolamine) from Avanti Polar Lipids is dissolved in chloroform/methanol 2:1 (v/v). Aliquots of 125 nmoles are dried in silanized 1.5-mL reaction tubes and stored at –20°C.
8. Chloroform/methanol 2:1 (v/v).
9. 1,1,-Dichloromethane.
10. Liposome buffer: 10 m*M* HEPES-NaOH, pH 6.5, 100 m*M* NaCl, 0.5 m*M* EDTA.

2.5. Floatation Assay

1. Assay buffer: 10 m*M* HEPES-NaOH, pH 7.0, 150 m*M* NaCl, 10 m*M* KCl, 2 m*M* $MgCl_2$, 0.2 m*M* DTT.
2. Sucrose solutions: 20% and 60% (w/v) in assay buffer.
3. 5’-Guanylylimidodiphosphate (GMP-PNP; Fluka) is prepared at 10 m*M* and GTP (Sigma) at 100 m*M* in water and stored at –80°C.
4. TCA: 100% (w/v) 2,2,2-trichloroacetic acid in water.
5. Acetone (analysis grade).

2.6. Sedimentation Assay

1. Gradient Master from BioComp Instruments (Fredericton) or alternative gradient maker.
2. Sucrose solutions: 10% and 25% (w/v) in assay buffer containing 0.2% (w/v) Triton X-100.
3. Triton X-100 at 20% (w/v) in water.

2.7. General Materials

1. Standard equipment and materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide) and immunoblot analysis.
2. Bradford Protein Assay (Bio-Rad) or equivalent.

3. Methods

For centrifugations, an example of rotors and speeds are provided in parentheses. Unless expressly stated, all procedures are performed at 4°C.

3.1. Isolation of Cytosol and CCVs

Intracellular membranes released from homogenized calf brains are recovered in a low-speed supernatant and separated from the cytosol by high-speed centrifugation. In a subsequent medium-speed centrifugation with Ficoll and sucrose, CCVs are enriched in the supernatant. After dilution, they are collected by high-speed centrifugation. The procedure is based on **ref. *12***.

1. Two calf brains fresh from the slaughterhouse are transported on ice to the coldroom (*see* **Note 3**).
2. Fat, brain stem, meningae, and blood clots are removed with paper towels.
3. The cleaned brains are placed in a beaker filled with 1 L PBS to measure their volume (~ 200 mL per brain).
4. The brains are placed in the Waring blender together with half their volume of buffer A (*see* **Note 4**). PMSF (0.5 mM) is added and the blender is turned on three times for 8 s on medium speed.
5. The homogenate is centrifuged at 8,000*g* for 30 min (Sorvall GS3 rotor, 7,000 rpm). Keep the pellets for **step 7**.
6. The supernatant is carefully collected (*see* **Note 5**) and the membranes are pelleted by centrifugation at 180,000*g* for 80 min (Kontron TFT 45.94 rotor, 40,000 rpm). The high-speed supernatant (i.e., the cytosol) is collected (*see* **Note 6**), aliquoted, shock-frozen in liquid nitrogen, and stored at –80°C. Protein concentration is typically 15 to 30 mg/mL. The high-speed pellets are kept for **step 8**.
7. The low-speed pellets of **step 5** are resuspended in an equal volume of buffer A and—after addition of 0.5 m*M* PMSF—re-homogenized in the blender as before

and centrifuged again at 8,000*g* for 30 min. The supernatant is collected and all membranes pelleted at 180,000*g* for 80 min. This second high-speed supernatant is discarded.

8. The high-speed pellets from **steps 6** and **7** are resuspended in 1 mL buffer A per tube with the help of a spatula, collected, and homogenized with five to six strokes in a dounce homogenizer.
9. The suspension (~8 mL) is mixed with an equal volume of buffer B, dounce homogenized again, and centrifuged at 60,000*g* for 40 min (Kontron TFT 45.94 rotor, 24,000 rpm; *see* **Note 7**).
10. The supernatants are collected, diluted with three volumes of buffer A, and centrifuged at 180,000*g* for 80 min (Kontron TFT 45.94 rotor, 40,000 rpm) to pellet the CCVs.
11. The pellets are resuspended in an equal volume of buffer A, homogenized in a small dounce homogenizer, and centrifuged in 1.5-mL tubes in a microfuge for 12 min to remove aggregated material.
12. The supernatant containing the CCVs is collected, frozen as 750-µL aliquots in liquid nitrogen, and stored at –80°C. Typically four aliquots are obtained from two brains.

3.2. Preparation of Mixed Adaptors

Clathrin coats are released from CCVs with high concentrations of Tris, and adaptors enriched by gel filtration. The procedure is based on **ref.** ***13***.

1. Two 750-µL aliquots of CCVs are thawed, mixed with an equal volume of stripping buffer containing 0.5 m*M* PMSF and 1X proteinase inhibitor cocktail in a small dounce homogenizer, and kept on ice overnight.
2. The mixture is dounce homogenized again and centrifuged at 100,000*g* for 35 min (Beckman TLA 100.3 rotor, 70,000 rpm) to pellet the membranes. If the supernatant is still turbid, it is centrifuged again.
3. Two milliliters of the clear supernatant are fractionated on the Superose 6 column pre-equilibrated in Superose running buffer at a flow rate of 0.5 mL per minute and collected in 1-mL fractions.
4. Samples of 25 µL of every second fraction from 41 to 72 are analyzed by SDS-gel electrophoresis and immunoblotting for the presence of AP-1 and clathrin. AP-1 is typically found in fractions 55 to 63 (*see* **Note 8**).
5. AP-1-containing fractions are pooled, supplemented with 1X protease inhibitor cocktail and 0.5 m*M* PMSF, and may be stored at 4°C for several weeks. Typically 10 mL at 100 µg/mL protein are obtained.

3.3. Preparation of Myristoylated Arf1

Arf1 and NMT are expressed in bacteria and myristic acid is exogenously provided to produce efficiently myristoylated Arf1. The protein is purified from freeze-thawed cells by ion exchange chromatography and gel filtration. The procedure is based on **ref.** ***14***.

1. Competent BL21(DE3) bacteria are mixed with approx 0.1 μg each of pET-mArf1* and pBB131 encoding Arf1 and NMT, respectively, incubated on ice for 1 h, and heat-shocked at 42°C for 2 min.
2. The bacterial suspension is incubated with 1 mL LB at 37°C for 1 h before addition of 4 mL LB with 100 μg/mL each of ampicillin and kanamycin and incubation overnight with shaking to select for transformants carrying both plasmids.
3. The overnight culture is used to inocculate 2.5 L LB with 100 μg/mL each of ampicillin and kanamycin. The culture is grown until OD_{600} reaches 0.6 to 0.8.
4. Arf1 and NMT expression is induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Simultaneously, 0.5% (w/v) Brij 58 (to increase the solubility of myristic acid) and 500 μ M myristic acid are added, and the culture is incubated 4 h at 30°C (a temperature that favors myristoylation).
5. The cells are harvested by centrifugation at 5,000*g* for 15 min (Sorvall GS3 rotor, 5,400 rpm).
6. The pellets are resuspended with PBS, transferred into a 50-mL Falcon tube, and pelleted again for freezing at –80°C.
7. To the frozen pellet (~5 mL), 25 μL 0.5 *M* PMSF and 50 μL 500X protease inhibitor cocktail are added, and the bacteria are lysed by three cycles of freezing in dry ice/methanol and thawing in luke warm water.
8. Bacteria are resuspended in 25 mL DEAE buffer and incubated on ice for 1 h before centrifugation at 20,000*g* for 15 min (Sorvall SS34 rotor, 13,000 rpm).
9. The supernatant is collected and loaded directly onto the 25-mL DEAE Sephacel column equilibrated and run with DEAE buffer at a flow rate of 1 mL per minute.
10. The flow-through contains Arf1 and is collected until OD_{280} is below 1.0.
11. These Arf1 containing fractions are concentrated by ultrafiltration (Amicon Ultra filter) to approx 2 mL.
12. The sample is fractionated at a flow rate of 1 mL per minute on the Superdex 75 column equilibrated with Superdex running buffer. Fractions of 3 mL are collected.
13. Fractions 51 to 80 are tested for the presence of Arf1 and contaminating proteins by analyzing 25-μL samples on parallel 15% SDS-gels by immunoblotting and Coomassie staining, respectively.
14. Arf1 containing fractions (typically fractions 60–70) are pooled (*see* **Note 9**) and concentrated to 1 mg/mL by ultrafiltration.
15. The purified protein is aliquoted, shock-frozen in liquid nitrogen, and stored at –80°C. Approximately 0.5 mg Arf1 is typically purified from a 2.5 L culture.

3.4. Preparation of Peptidoliposomes

Liposomes are prepared by extrusion through a defined pore-size filter. A maleimide-containing lipid is included to allow chemical coupling of cysteine-terminal peptides. The procedure is based on **refs.** ***6*** **and** ***15***.

1. Five µmoles of the desired lipids, for example 3.8 mg egg PC, soybean lipids, or a 1:1 mixture of the two, are dissolved in 1 mL chloroform/methanol in a test tube that can stand liquid nitrogen.
2. 125 nmoles (2.5 mol %) MMCC-DOPE is dissolved in 100 µL chloroform/methanol and added to the lipids (see **Note 10**).
3. The organic solvent is evaporated under a stream of nitrogen
4. The lipids are redissolved in 2 mL of 1,1-dichloromethane and dried again under nitrogen.
5. Meanwhile, the extruder is assembled and washed with liposome buffer, and 0.28 µmoles (400 µg) of the peptides to be coupled (approximately fourfold excess over the reactive lipid, assuming half of it is exposed to the outside of the liposome) are weighed out and dissolved in 100 µL liposome buffer.
6. The dried lipids are suspended in 1 mL liposome buffer by five cycles of vortexing, shock-freezing in liquid nitrogen, and thawing under warm tap water (*see* **Note 11**).
7. Lipids are passed 11 times through the extruder unit (*see* **Note 12**).
8. The liposomes are immediately mixed with 100 µL 4 mg/mL peptide and incubated for 1 h at room temperature (*see* **Note 13**).
9. The final peptidoliposomes are stored at 4°C with 0.02% (w/v) NaN_3 for up to 2 wk (*see* **Notes 14** and **15**).

3.5. Floatation Assay

Peptidoliposomes are incubated with coat proteins, Arf1, and nucleotides to allow for Arf1 activation and coat recruitment, and then floated on a sucrose step gradient. Proteins associated with the liposomes or remaining in the loading zone are collected by trichloroacetic acid (TCA) precipitation and detected by immunoblot analysis. The procedure is based on **refs. *6*** and ***9***.

1. Cytosol is centrifuged at 170,000*g* for 30 min (Beckman TLA100.3 rotor, 85,000 rpm) to remove aggregates.
2. Peptidoliposomes (100 µL containing ~0.5 µmol lipid) are mixed with 5 µg Arf1, 0.2 m*M* GMP-PNP or 2 m*M* GTP, either 10 µg mixed adaptors or 1 mg cytosol, and reaction buffer to 200 µL. The mixture is incubated for 30 min at 37°C to allow nucleotide exchange on Arf1 and protein recruitment.
3. The reaction is mixed in a 4-mL ultracentrifuge tube with 0.4 mL 60% sucrose solution (to a final concentration of 40% sucrose). The mixture is carefully overlayed with 1 mL of 20% sucrose solution. After removal of any foam at the top with a pipet, 20% sucrose solution is added to a total of 3.82 mL.
4. The tubes are balanced and 180 µL assay buffer is overlayed to facilitate liposome collection afterwards.
5. Samples are centrifuged at 300,000*g* for 1 h (Kontron TST60.4 rotor, at 55,000 rpm).
6. Four 1-mL fractions are collected from the top.

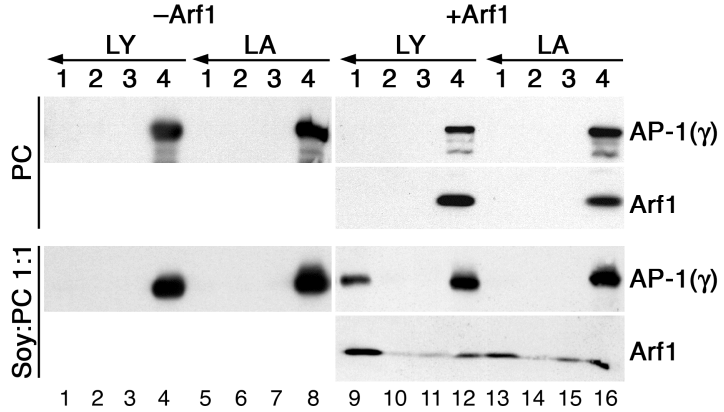

Fig. 1 AP-1 recruitment to peptidoliposomes is Arf1-, lipid-, and signal-dependent. Peptidoliposomes made of 100% PC or a 1:1 PC/soybean lipid mixture and presenting LY or LA peptides were incubated with mixed clathrin adaptors and with or without Arf1 and GMP-PNP. After flotation on a sucrose step gradient, four fractions were collected from the top and analyzed by immunoblotting for γ-adaptin (a subunit of AP-1) or Arf1. Recruitment of AP-1 required Arf1, lipids other than PC, and the presentation of the Lamp1 sequence with an intact tyrosine motif (LY). While Arf1 is activated on PC membranes (not shown), it also requires non-PC lipids for binding to liposomes. Arrows indicate the direction of floatation from the bottom to the top fraction of the gradient. (Reprinted in modifed form from **ref. *6*** with permission of The American Society for Cell Biology.)

7. TCA precipitation: the fractions are mixed with 160 μL TCA in 1.5-mL tubes and centrifuged in a microfuge at maximal speed for 15 min.
8. To the pellets, 850 μL ice-cold acetone is added, and the tubes are centrifuged again for 5 min at 4°C.
9. The pellets are air-dried for 15 to 20 min at room temperature and dissoved in 60 μL SDS-sample buffer by pipeting up and down and by heating at 95°C for 10 min (*see* **Note 16**).
10. Samples are separated by gel electrophoresis on a 12.5% SDS-gel and analyzed by immunoblotting for AP-1 (γ-adaptin) and Arf1. An example result is shown in **Fig. 1** (and using cytosol in **Fig. 3**, left lanes).

3.6. Sedimentation Assay

To analyze the oligomerization state of liposome-associated protein, membranes in the floated fraction are solubilized and the protein centrifuged into a sucrose density gradient. Protein in the gradient fraction is detected by immunoblot analysis. The procedure is based on **ref. *9***.

1. The top 350 μL of a floatation gradient (**Subheading 3.5**, **step 5**), which contain most of the floated liposomes, are collected and mixed with 350 μL assay buffer to dilute the sucrose.
2. The lipid membranes are solubilized by addition of 0.5% Triton X-100.

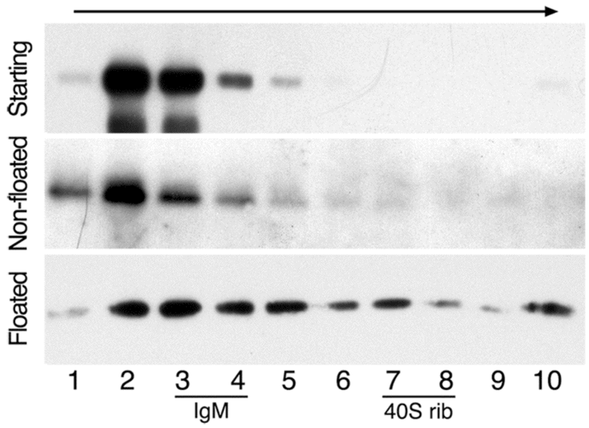

Fig. 2 AP-1 recruited to peptidoliposomes forms high-molecular weight complexes. Peptidoliposomes made of soybean lipids and presenting LY peptides were incubated with clathrin adaptors, Arf1, and GMP-PNP. After floatation on a sucrose step gradient, the floated liposomes were solubilized with Triton X-100 and centrifuged into a 10 to 25% sucrose velocity gradient (horizontal arrow). Ten fractions were collected and analyzed by immunoblotting for γ-adaptin. For comparison, the nonfloated fraction and the original adaptors were analyzed in parallel. The positions of the sedimentation markers IgM (19S) and 40S ribosomes are indicated. Individual AP-1 adaptors (~300 kD) have a sedimentation coefficient of 7.7S. AP-1 recruited to sorting peptides show oligomerization to high-molecular-weight complexes even in the absence of clathrin. (Reprinted in modifed form from **ref.** ***9*** with permission of The American Society for Cell Biology.)

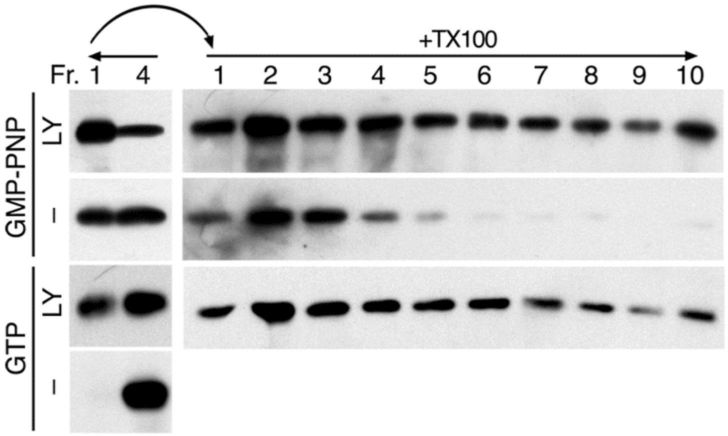

Fig. 3 AP-1 recruitment from cytosol. Bovine brain cytosol was supplemented with Arf1, 0.2 m*M* GMP-PNP or 2 m*M* GTP, and liposomes with or without LY peptides as indicated and incubated for 30 min at 37°C. After a first step gradient floatation, the floated fraction 1 was solubilized with Triton X-100 and sedimented into a sucrose gradient as in **Fig. 2**. The fractions were analyzed by immunoblotting for γ-adaptin. Unlike AP-1 from purified adaptors, AP-1 from cytosol can be recruited to liposomes with Arf1/GMP-PNP even in the absence of sorting peptides. However, LY peptides are required for AP-1 to oligomerize. If GTP is used, stable recruitment is observed only with sorting peptides. These results indicate the presence of a cytosolic factor recruiting

3. The sample is loaded onto a 4.3-mL 10 to 25% sucrose gradient with 0.2% Triton X-100 and centrifuged at 100,000*g* for 5 h (Kontron TST 55.5 rotor, at 30,000 rpm).
4. Ten 0.5-mL fractions are collected from the top and 125 µL of 5X SDS-sample buffer are added for analysis by SDS-gel electrophoresis and immunoblotting for AP-1 (γ-adaptin). An example result is shown in **Figs. 2** and **3**.

4. Notes

1. Alternatively, 10 pig brains may be used.
2. Ficoll 400 is hard to dissolve. Warm up the solution and add Ficoll 400 step by step with continued stirring.
3. To process two brains, it is convenient in the following procedure to use two TFT45.94 rotors and two ultracentrifuges in parallel. For upscaling to six brains (2 × 3 brains), one needs two GS3 and three TFT45.94 rotors and appropriate centrifuges.
4. The buffer volume is kept low to retain high cytosol concentration.
5. It is really important to only take the red supernatant. Pour the supernatant over the white pellet into a beaker. It is better to take less supernatant than to have contamination with material from the pellet.
6. It is important not to contaminate the cytosol with the pellet. Carefully remove the cytosol with a glass pipet, leaving behind the last 1 cm of supernatant above the pellet.
7. To fill tubes to their minimal filling level or to balance them, a 1:1 mixture of buffers A and B may be added.
8. AP-2, AP180, and some clathrin are the major contaminants.
9. The later fractions of the Arf1 peak are usually purer than the earlier ones.
10. In addition, (*N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine [NBD-PE]; from molecular probes), a fluorescent phospholipid analog, may be added at 1 mol% to quantify liposome recovery in the subsequent experiments by fluorimetry (λ_{ex} 450 nm, λ_{em} 530 nm).
11. As soon as the liposome buffer is added to the dried lipids it is important to work quickly because of hydrolysis of the maleimide group.
12. Be very careful during the extrusion not to break the membranes. If you are stuck, back up a little before pushing on. After extrusion, disassemble the extruder and make sure the filter is intact. If the filter is damaged, repeat the extrusion.
13. To determine coupling efficiency, *see* **ref. *16***. The coupling reaction at pH 6.5 is essentially complete within 10 min at room temperature ***(16)***.

◄

Fig. 3. (Continued) AP-1 to membranes in a cargo-independent, GTPase-sensitive manner. They further show that membrane recruitment is not sufficient to induce AP-1 oligomerization, but that binding to cargo signals is necessary. (Reprinted in modifed form from **ref. *9*** with permission of The American Society for Cell Biology.)

14. Some peptides induce liposome aggregation with time, which may cause unspecific trapping of protein in the floatation assay. These liposomes have to be used more quickly or even on the same day only.
15. For most uses, the free, excess peptides do not have to be removed (e.g., by gel filtration or dialysis), because the concentration in solution is much lower than that at of coupled peptides at the membrane surface.
16. Especially when using cytosol, pellets from the bottom fraction may be hard to dissolve. Also, if samples turn yellow, the pH can be adjusted by pipeting ammonia vapour onto them.

Acknowledgments

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Results-Part II

A cytosolic factor mediating membrane recruitment of AP-1 clathrin adaptors

An assay to follow the activity of the cytosolic factor

Previous in vitro studies have shown that the minimal requirements to recruit AP-1 to liposomal membranes are activated Arf1, specific phosphoinositides and either a tyrosine based sorting signal or an unknown cytosolic docking factor (Crottet et al., 2002; Zhu et al., 1999b). In order to purify and thus identify this unknown factor we used a liposome recruitment assay illustrated in Figure 1.

Soybean liposomes (consisting of an ill-defined lipid mixture including phosphoinositides and 20% phosphatidylcholine) were incubated at 37°C for 30 min with recombinantly produced myristoylated Arf1-His$_6$, a nonhydrolyzable GTP analog (GMP-PNP) and AP-1. Under these conditions AP-1 is only recruited if the cytosolic factor is present. As a positive control the presence of cargo proteins was mimicked by coupling a synthetic peptide corresponding to the cytosolic domain of Lamp-1 (lysosome-associated membrane protein-1) to the liposomes by the lipid reagent MMCC-DOPE. Lamp1 contains a well characterized YXXΦ motif which was shown to be sufficient to recruit AP-1 to soybean liposomes in the presence of activated Arf1 (Meyer et al., 2005). To separate the liposomes and bound proteins from non-recruited material the mixture was supplemented with sucrose to a final concentration of 40% (wt/vol) and overlaid with 30% (wt/vol) sucrose and a small amount of sucrose-free assay buffer before it was centrifuged for 1.5 h at 300’000 x g (Figure 1A). The top fraction (designated #1) containing the floated liposomes together with recruited material, was collected and analyzed for the presence of AP-1 with SDS-gel electrophoresis and immunoblotting.

When we used purified AP-1 that was released from calf brain CCVs with 1 M Tris followed by gel filtration, AP-1 recruitment was strictly dependent on the presence of cargo peptides (Figure 1B, lanes 1 and 2). When full cytosol was used as a source of AP-1, however, it could also be recruited in the absence of sorting signals (lanes 3 and 4), indicating the presence of the cytosolic docking factor.

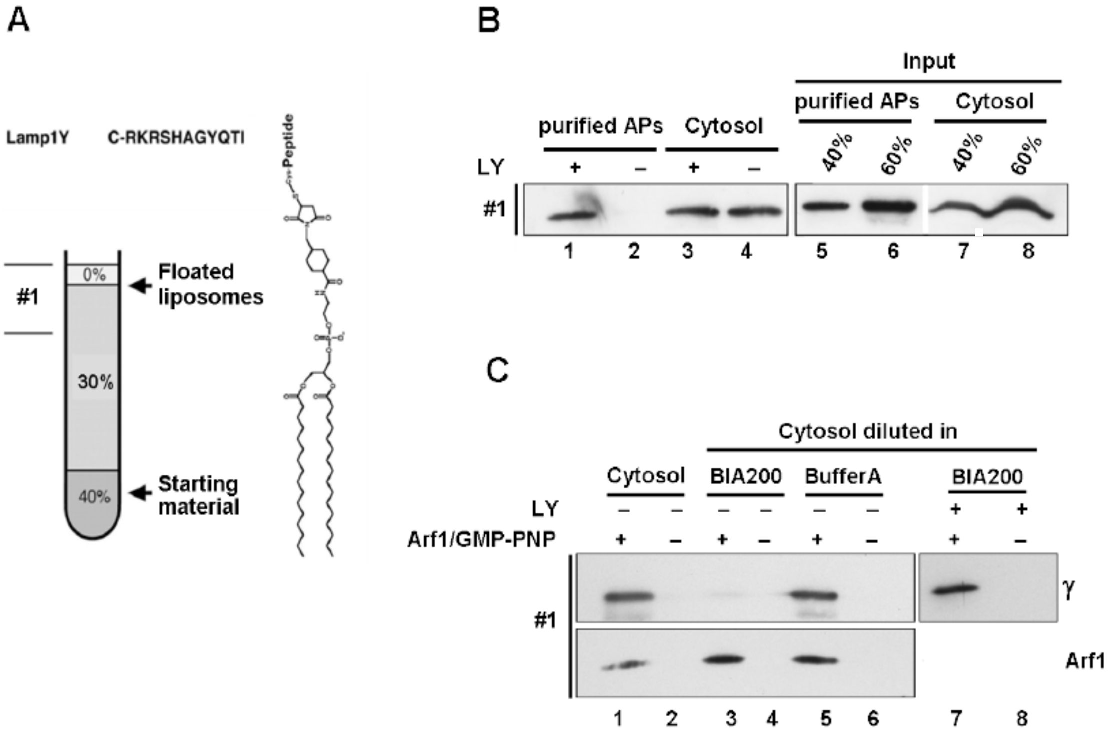

Figure 1: **The liposome recruitment assay.**

(A) Samples were tested for the presence of the cytosolic factor using a recruitment assay to follow AP-1 recruitment to liposomes. Samples were incubated for 30 min at 37°C with 100 µl soybean liposomes (0.5 µmol lipid), 2 µg Arf1-His$_6$, 0.2 mM GMP-PNP and 10 µg of purified coat derived adaptors before the mixture was loaded on the bottom of a sucrose step gradient and centrifuged at 300’000 x g for 1.5 h. The top fraction (#1) containing the floated liposomes with bound proteins was collected and analyzed by immunoblotting for γ-adaptin (AP-1). As a positive control a maleimide derivate of PE MMCC-DOPE was used to couple a synthetic peptide (Lamp1Y), via an N-terminal cysteine to a lipid. The peptide corresponds to the cytoplasmic domain of Lamp1. (B) Liposomes with or without LY signal peptide were incubated with Arf1-His$_6$, GMP-PNP and either 1 mg calf brain cytosol or 10 µg purified adaptors. After flotation the top fraction was analyzed by immunoblotting for γ-adaptin (AP-1). Lanes 5-8 represent 40% and 60% of the starting material: either purified adaptors (lanes 5 and 6), or cytosol (lanes 7 and 8). (C) 660 µl calf brain cytosol was diluted in either BIA200 buffer (lanes 3, 4, 7 and 8) or BufferA (lanes 5 and 6) to a final volume of 2 ml. 330 µl of this mixture was then incubated with liposomes with or without LY peptides and with or without Arf1-His$_6$ and GMP-PNP. After flotation the floated liposomes and bound proteins were analyzed by immunoblotting. As a positive control 110 µl of undiluted cytosol was also subjected to the recruitment assay (lanes 1 and 2).

Recruitment of AP-1 by the docking factor was strongly dependent on buffer conditions in the recruitment assay (Figure 1C). When cytosol was diluted three times in a high salt buffer (BIA200 buffer; 18.5 mM Hepes pH 7, 244 mM NaCl, 15 mM KCl, 3 mM MgCl$_2$, 0.2 mM DTT) no AP-1 recruitment could be detected (lane 3). This was not an effect of dilution, since cytosol diluted in assay buffer (BufferA; 0.1 M MES/NaOH, pH 6.6, 0.5 mM MgCl$_2$, 1 mM EGTA, 0.2 mM DTT) was still able to recruit AP-1 (compare lanes 3 and 5), suggesting that the interaction of AP-1 with the factor is highly salt sensitive. Interestingly, the high salt buffer did not affect AP-1 recruitment to peptidoliposomes that contained the LY sorting signal (lane 7). This is in agreement with Meyer et al. (2005) who demonstrated that in contrast to the docking factor, signal tails lead to AP-1 oligomerization which increases the avidity of AP-1 to membranes through multiple low affinity bindings.

Both cytosolic and coat derived AP-1 require a cytosolic factor

AP-1 undergoes a complex cycle of phosphorylation and dephosphorylation at the β_1 and μ_1 subunits in vivo. Cytosolic AP-1 was shown to be selectively phosphorylated on the hinge of its β_1 subunit (Wilde and Brodsky, 1996). Upon membrane recruitment β_1 is dephosphorylated whereas the μ_1 of AP-1 undergoes phosphorylation which results in a conformational change of AP-1 (Ghosh and Kornfeld, 2003a). The difference in behaviour in in-vitro recruitment between coat derived purified AP-1 and cytosolic AP-1 could thus simply reflect different conformation states.

In order to address this question, AP-1 was either immunopurified from cytosol or released from calf brain CCVs like in the previous experiment. Figure 2A shows aliquots of these individual preparations loaded on a SDS-gel and stained with Coomassie. When the two AP-1 sources were tested in the recruitment assay, they were unable to be recruited to soybean liposomes in the absence of signal tails and cytosol (Figure 2B, lanes 1 and 4). To demonstrate that the lack of AP-1 recruitment was not due to processes involved in its purification, calf brain cytosol was immunodepleted of endogenous AP-1 before it was incubated at 37°C for 30 min with either cytosol or coat derived AP-1, Arf1-His$_6$, GMP-PNP and liposomes. Immunoblotting of the floated fractions demonstrated that in the presence of the cytosolic factor, both the coat and cytosol derived AP-1 recruited to soybean liposomes in similar amounts (lanes 5 and 6). Thus, despite different conformations, both coat derived and cytosolic AP-1 need a cytosolic factor to be recruited to membranes in the absence of signal tails. Furthermore, the contaminant proteins that were co-isolated with AP-1 from calf brain cytosol or CCVs (Figure 2A) seem not to be sufficient to mediate AP-1 recruitment in the absence of cargo.

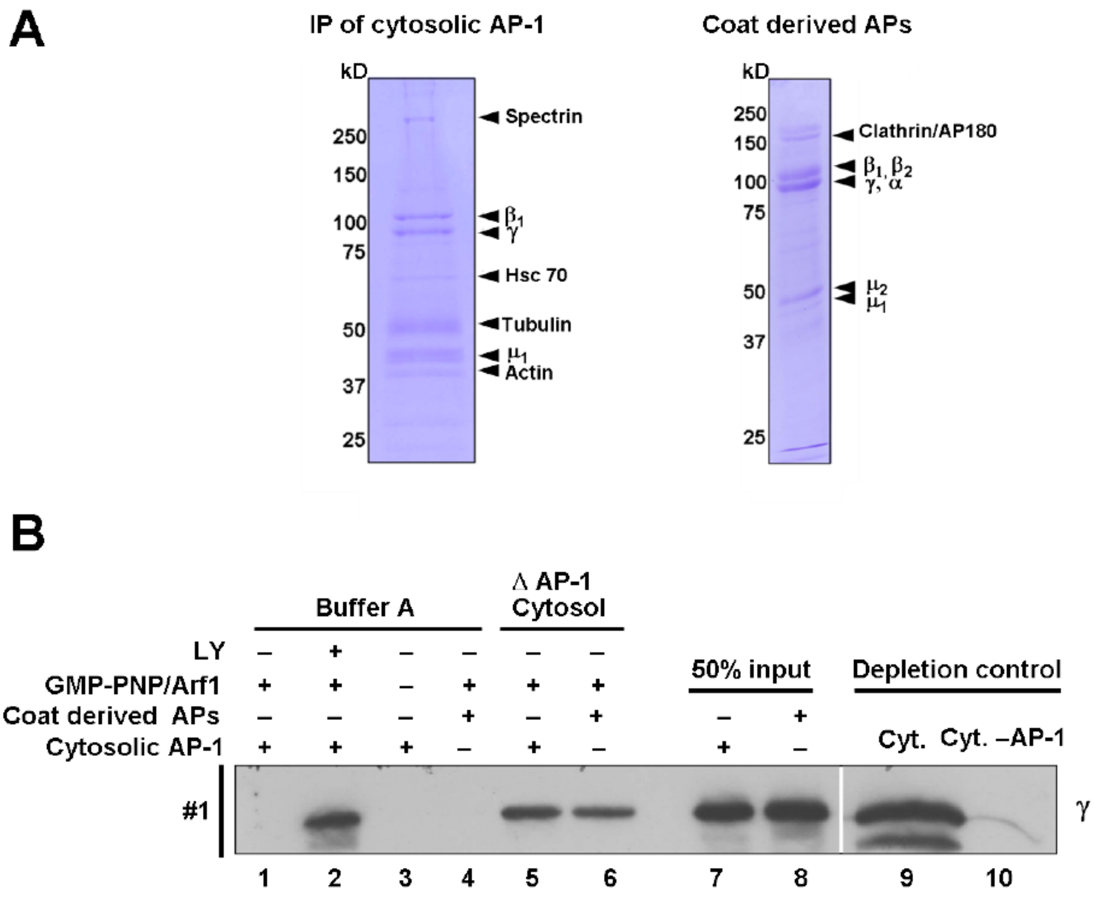

Figure 2: **Coat derived and cytosolic AP-1 need a cytosolic factor:**

(A) Aliquots of immunopurified cytosolic AP-1 and of the mixed adaptor preparation were separated by SDS-gel electrophoresis and stained with Coomassie. AP-1 subunits and contaminating proteins, that were identified by mass spectrometry, are indicated by arrowheads. (B) Liposome recruitment assays were performed, using either cytosolic AP-1 or the mixed adaptor preparation. As a positive control, liposomes containing the LY signal peptide were used (lane 2). The negative control (lane 3) contained no Arf1-His$_6$ and GMP-PNP. 5 mg of AP-1 depleted cytosol served as a source for the docking factor to test the functionality of the two AP-1 preparations (lanes 5 and 6). Lanes 7 and 8 represent 50% of the AP-1 input. The doublet observed on the blot in lane 9 and in some of the subsequent experiments, is the consequence of partial proteolysis of the γ appendage domain of the γ subunit of AP-1.

The docking factor is a protein

In order to address the nature of the docking factor, cytosol was incubated with 1,1,2-trichloro-trifluoroethane to remove potentially remaining lipids before it was supplemented with coat derived APs, Arf1-His$_6$ and GMP-PNP and tested in our assay for its ability to recruit AP-1 to soybean liposomes (Figure 3). Delipidation of the cytosol did not result in the removal of the factor, since AP-1 was still recruited (lane 6). Thus the factor is not a lipid. In contrast, when cytosol was treated with proteinase K prior to the flotation assay, AP-1 recruitment was abolished to background levels (lanes 4 and 5). This was not due to incomplete removal of the protease prior to the assay, since Arf1-His$_6$ was fully intact and showed robust membrane recruitment. The stronger Arf1 signal observed in lanes 2, 3 and 6 was due to additional cytosolic Arf1 that was present in these samples which was not removed prior to the recruitment assay. Previous experiments showed that Arf1 is not a limiting factor in the assay (data not shown) thus together theses data demonstrate that the cytosolic factor is a protein.

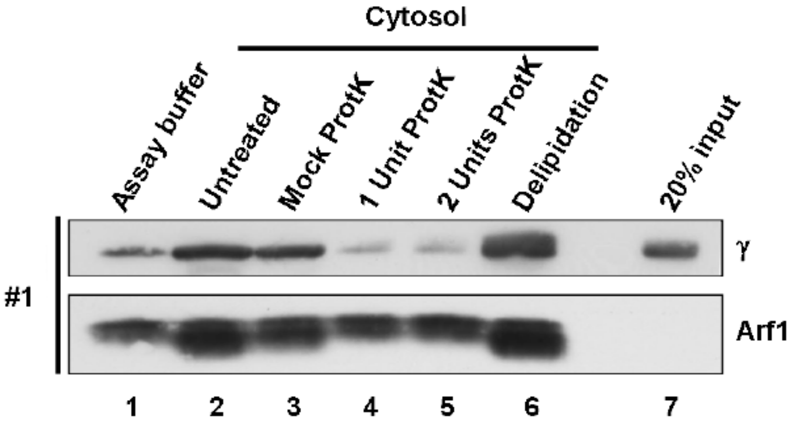

Figure 3: **The docking factor is a protein:**
1 mg of calf brain cytosol was either incubated for 1 h at 37°C with (lanes 4 and 5) or without (lane 3) indicated amounts of proteinase K (ProtK) or incubated for 1.5 h at room temperature with 1,1,2-trichloro-trifluoroethane (lane 6). After removal of the proteinase and remaining lipids, the samples were supplemented with Arf1-His$_6$, GMP-PNP, mixed adaptors and soybean liposomes in order to do a recruitment assay. 1 mg of untreated cytosol served as a positive control (lane 2) whereas the negative control contained assay buffer instead of cytosol. No additional AP-1 was added to the untreated cytosol (lane 2) and to the mock treated cytosol (lane 3) during the recruitment assay, since they contained sufficient cytosolic AP-1. Lane 7 represents 20% of the mixed adaptors input.

The factor is recruited to membranes in an Arf1 independent manner

The cytosolic docking factor was shown to be a peripheral membrane protein that strongly associated with membranes (Zhu et al., 1999b). However, nothing is known about the mechanism of its membrane recruitment. Since it was proposed that the factor interacts at the membrane with activated Arf1 to produce high affinity binding sites for AP-1 (Zhu et al., 1998), we wanted to test if membrane recruitment of the factor is Arf1 dependent.

To answer this question, a two-stage flotation assay depicted in Figure 4A was used. In the first stage the cytosolic factor was allowed to recruit to soybean liposomes from AP-1 depleted cytosol in the presence or absence of Arf1-His$_6$ and GMP-PNP. After a 30 min incubation at 37°C and subsequent flotation, the liposomes were collected. To test whether the factor was recruited in an Arf1 dependent manner during the first stage, collected liposomes were subjected to a second recruitment assay where no additional cytosolic factor was added. Liposomes where incubated with purified AP-1 (either from cytosol or CCVs) and BSA (to block unspecific binding) and supplemented or not with Arf1-His$_6$ and GMP-PNP. After flotation liposomes were collected and tested for the presence of AP-1 by immunoblotting.

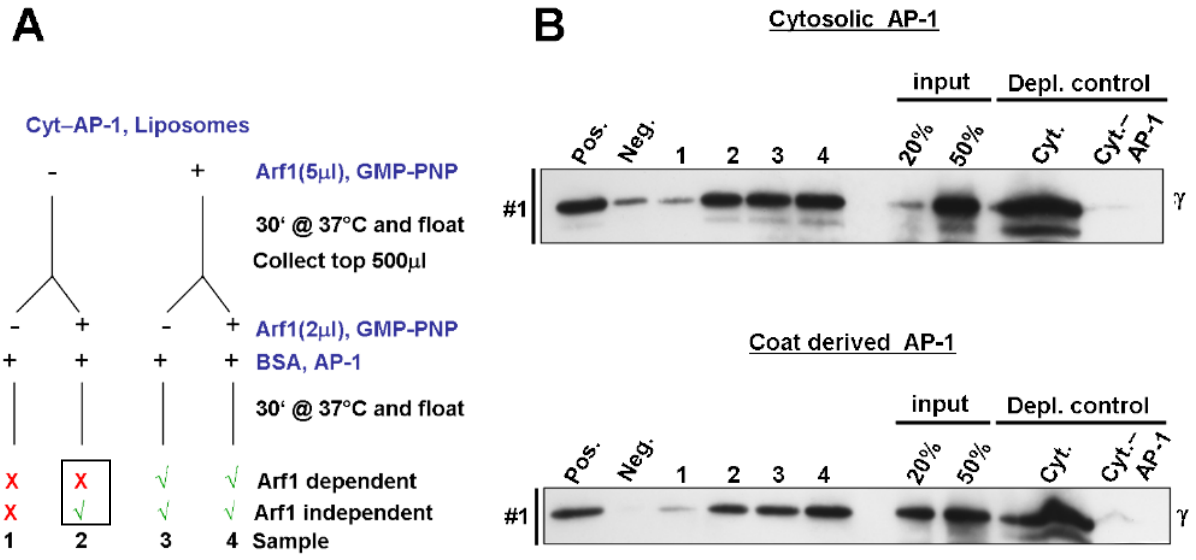

Figure 4. **Recruitment of the cytosolic factor is Arf1 independent:**
(A) 5 mg of AP-1 depleted cytosol was supplemented with liposomes and either Arf1-His$_6$ and GMP-PNP or not, and incubated for 30 min at 37°C. After a first step gradient flotation the floated liposomes were collected and subjected to a second flotation after the addition of AP-1, BSA and either Arf1 and GMP-PNP or not. (B) The collected liposomes from the second flotation were analyzed by immunoblotting for γ-adaptin. Experiments were performed with AP-1 that was either immunopurified from cytosol, or hydroxyapatite-purified from CCVs. As a positive control (Pos.) AP-1 depleted cytosol was supplemented with AP-1, liposomes and activated Arf1-His$_6$ before a recruitment assay was performed. The negative control (Neg.) contained assay buffer instead of cytosol. Right: controls.

No AP-1 recruitment was detected when Arf1 was absent in both stages (sample 1), consistent with the fact that activated Arf1 is essential for AP-1 binding. However, as soon as Arf1 was present in the assay, AP-1 recruitment could be observed (samples 2-4), demonstrating that the cytosolic factor was stably recruited to soybean liposomes during the first stage. AP-1 binding was also detected when Arf1 was only added during the second stage (sample 2) demonstrating that the factor was recruited in an Arf1 independent manner.

This was further supported in another two-stage assay (Figure 5), where soybean liposomes were incubated in the first step with calf brain cytosol at two different temperatures to test if membrane recruitment of the factor is temperature sensitive. After flotation, liposomes were collected and in a second step incubated at 37°C with AP-1, Arf1-His$_6$, GMP-PNP and BSA before they were floated again and analyzed by immunoblotting (Figure 5A). AP-1 recruitment was observed independent of the temperature the liposomes were incubated at during the first stage, indicating that recruitment of the factor occurs also at 4°C (Figure 5B, lanes 1 and 2). This is in agreement with our finding that membrane association of the factor is Arf1 independent, since Arf1 is not activated and recruited to soybean liposomes at 4°C (Crottet et al., 2002).

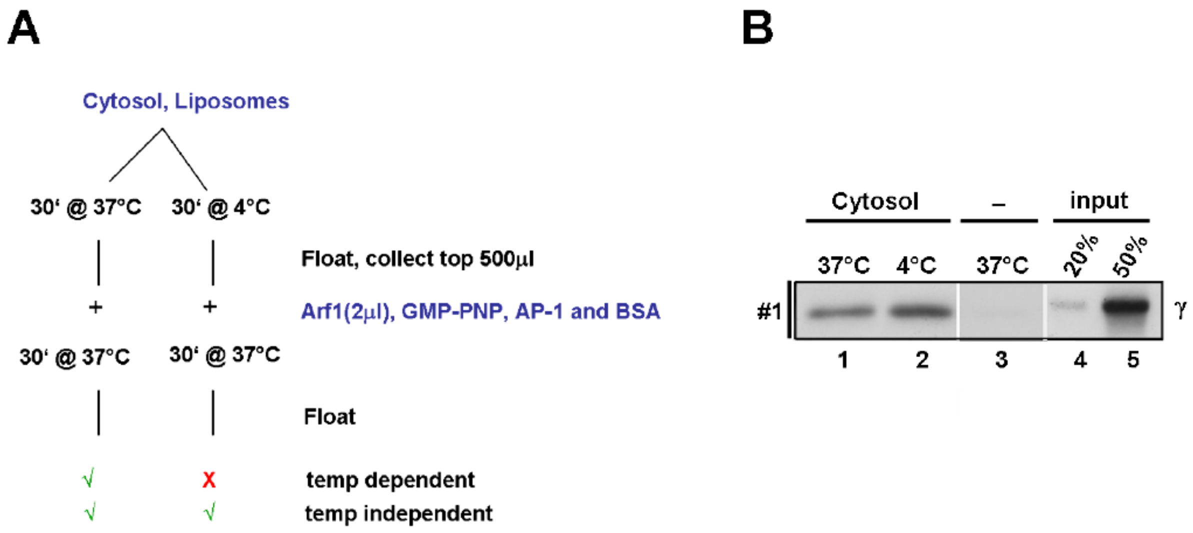

Figure 5. **Recruitment of the factor is temperature independent:**
(A) 5 mg of calf brain cytosol was supplemented with liposomes and incubated for 30 min at either 4°C or 37°C. After a first flotation, the top fraction was collected and incubated for 30 min at 37°C with Arf1-His$_6$, GMP-PNP, mixed adaptors and BSA. (B) The floated liposomes from the second recruitment assay were loaded on an

SDS-gel and analyzed by immunoblotting. As a negative control assay buffer was used instead of cytosol. Lanes 4 and 5 represent indicated percentages of AP-1 input.

γ-BAR is not the cytosolic factor

In a recent publication a novel protein has been described to have a role in AP-1 recruitment (Neubrand et al., 2005). The ~30 kD protein called γ-BAR was shown to be a peripheral membrane protein that colocalized with AP-1. Its overexpression specifically increased AP-1 membrane association and protected AP-1 from membrane dissociation after BFA treatment (see introduction).

In order to test if γ-BAR is responsible for cargo independent AP-1 recruitment, calf brain cytosol was fractionated on a sizing column. After immunoblotting of the eluted fractions γ-BAR was mainly found in fractions 76-80, whereas the tetrameric adaptor protein AP-1 eluted in fractions 52-64 (Figure 6A). In order to test these fractions for recruitment activity, cytosolic AP-1 had to be depleted (to ensure that every fraction had the same amount of supplemented AP-1), prior to the recruitment assay. Figure 6B shows that the factor was mainly present in fractions 46-58 which contained proteins that were even bigger than AP-1 (~260 kD). Thus γ-BAR could not be the cytosolic factor we were looking for.

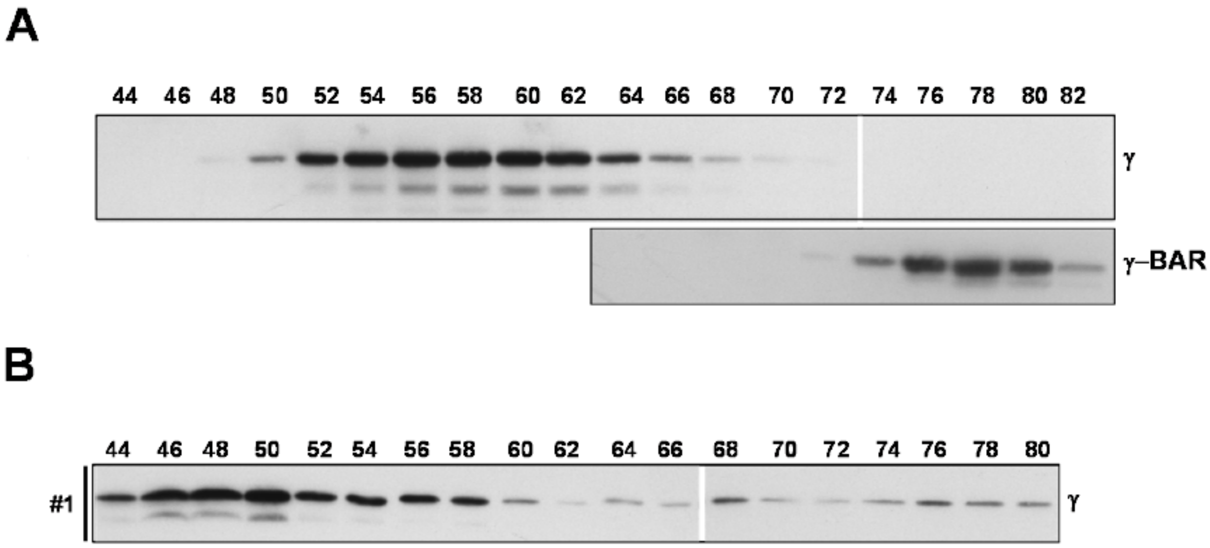

Figure 6. **The cytosolic factor is bigger than AP-1:**
(A) 60 mg of prespun calf brain cytosol was loaded on a Superdex 200 sizing column and 1 ml fractions were collected. Every other fraction was separated by SDS-gel electrophoresis and analyzed by immunoblotting. (B) 60 mg of AP-1 depleted calf brain cytosol was separated on a Superdex 200 sizing column. 330 µl of the collected 1 ml fractions were used in the recruitment assay to test for the presence of the cytosolic factor. Floated fractions were analyzed by immunoblotting.

Purification of the cytosolic factor

To purify the cytosolic docking factor, calf brain cytosol was fractionated using a combination of different protein purification methods. In all these individual purification steps the activity of the factor was followed with the liposome recruitment assay. Since we observed that this assay is very sensitive to the buffer composition of the sample, it was of great importance to always change a given sample buffer to assay buffer prior to the recruitment assay. This was achieved by ultrafiltration. Since the factor had an apparent size of more than 260 kD in gel filtration, we used a filtering device with a molecular weight cut-off of 50 kD. The concentrated material was then diluted in assay buffer and used in the recruitment assay. Hence, the ultrafiltration step was not only used to change the buffer of the sample but also to remove small proteins.

Ammonium sulfate precipitation is commonly used a first purification step to select for proteins of similar solubility, provided activity is retained (Figure 7). To test that and to determine at which ammonium sulfate concentration it precipitates, 20 ml of cytosol was supplemented with a saturated ammonium sulfate solution to a final concentration of 10% saturated (sat.) ammonium sulfate and incubated for 1 h at 4°C, before precipitated proteins were collected by centrifugation. In order to test the pellet for the presence of the factor, it was dissolved in assay buffer and loaded on a PD-10 desalting column to remove the ammonium sulfate which might interfere with the recruitment assay. The supernatant of the 10% (sat.) ammonium sulfate precipitation, on the other hand, was supplemented with ammonium sulfate to a final concentration of 30% (sat.) and incubated for 1 h at 4°C. Again precipitated proteins were collected and prepared for the recruitment assay whereas the supernatant was supplemented with ammonium sulfate to 50% (sat.) final concentration. This time both the pellet and the supernatant were desalted with PD10 columns and analysed in the recruitment assay. As a positive control either untreated cytosol or peptidoliposomes presenting the LY signal peptides were used. Normal assay buffer was used as a negative control. As shown in Figure 7A the factor still showed activity after ammonium sulfate precipitation. The pellet of the 10-30% (sat.) ammonium sulfate precipitation contained most of the activity (compare lanes 2, 5 and 8), whereas the supernatant (P 30- 50% and S 50%) contained almost no activity but many other cytosolic proteins, as detected by SDS-gel electrophoresis and Coomassie staining (Figure 7B). Thus, with a 30% (sat.) ammonium sulfate precipitation the factor is concentrated and a lot of contaminating proteins are removed.

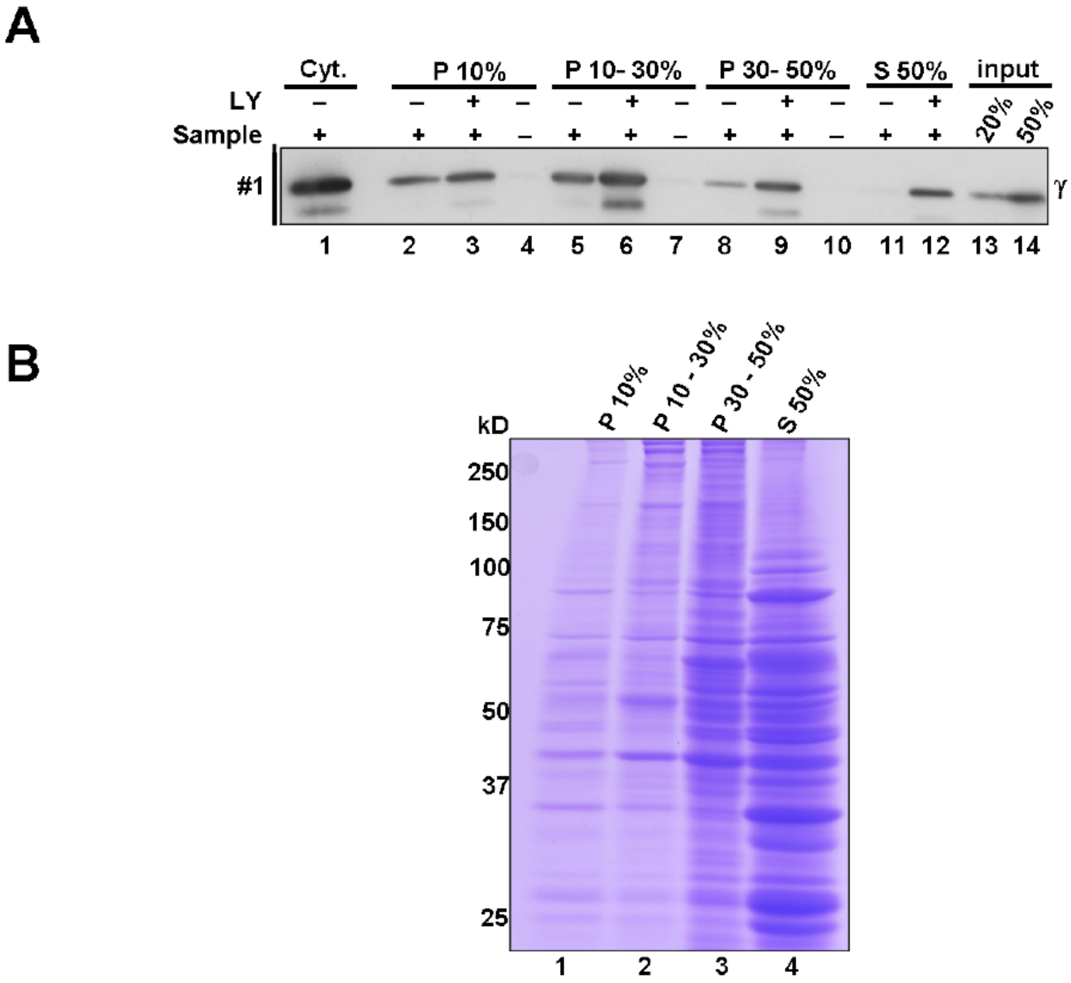

Figure 7. **The docking factor precipitates at 30% (sat.) ammonium sulfate:**
(A) 20 ml of calf brain cytosol (~70 mg) was supplemented with a saturated ammonium sulfate solution to different final concentrations of saturated ammonium sulfate (see text). The pellets of precipitated proteins (P) and the supernatant of a 50% (sat.) ammonium sulfate precipitation (S) were tested in a recruitment assay for the presence of the factor (lanes 2, 5, 8 and 11). As a positive control untreated cytosol or soybean liposomes with the coupled LY peptide were used (lanes 1, 3, 6, 9 and 12) whereas ammonium sulfate treated assay buffer instead of cytosol served as a negative control (lanes 4, 7 and 10). All recruitment assays were performed with 330 µl of the corresponding sample. The dissolved pellets and the supernatant fraction had a final volume of 5 ml. Lanes 13 and 14 represent different percentages of mixed adaptors input. (B) 20 µl of the samples were separated on SDS-PAGE and stained with Coomassie. It should be noted that the samples had different protein concentrations.

After ammonium sulfate precipitation, the factor was further purified by hydrophobic interaction chromatography (HIC). The interaction between proteins and the hydrophobic surface of the column is enhanced by high ionic strength buffers; therefore the factor-containing sample of a 30% (sat.) ammonium sulfate precipitation was supplemented with NaCl to a final salt concentration of 2 M NaCl prior to loading it onto the column. In order to separate proteins of different hydrophobicities from each other, they were eluted with a linear decreasing salt gradient of 200 ml from 2 M - 0 M NaCl. Collected fractions were then tested for recruitment activity after the buffer had been changed to assay buffer (Figure 8A). Most factor activity was observed in the fraction that contained the last 20 ml of the gradient (lane 12), suggesting that the factor strongly bound to the column and therefore is highly hydrophobic.

The factor-containing HIC fraction was then loaded on an anion exchange column (MonoQ) that was preequilibrated in assay buffer. However, since remaining salt from the HIC experiment would influence protein binding to the MonoQ column, the sample was desalted on a PD10 column prior to loading it onto the anion exchanger. Bound proteins where separated from each other according to their charge, by releasing them in a linear 80 ml gradient of increasing salt concentrations from 0-2 M NaCl. Thereafter, the buffer of the eluted fractions was changed to assay buffer, before samples where tested for the presence of the factor with the recruitment assay (Figure 8B). Most of the factor activity was found in the two fractions containing proteins that eluted between 29 ml and 36 ml of the gradient (lanes 8 and 9). Thus it seems that the factor is negatively charged at pH 6.6. However, there was also some activity in the flow through (FT) indicating that under the chosen conditions not all factor proteins were negatively charged (lane 4). To prevent contamination of the factor with proteins from the flow through we decided to collect only the two fractions that contained bound material.

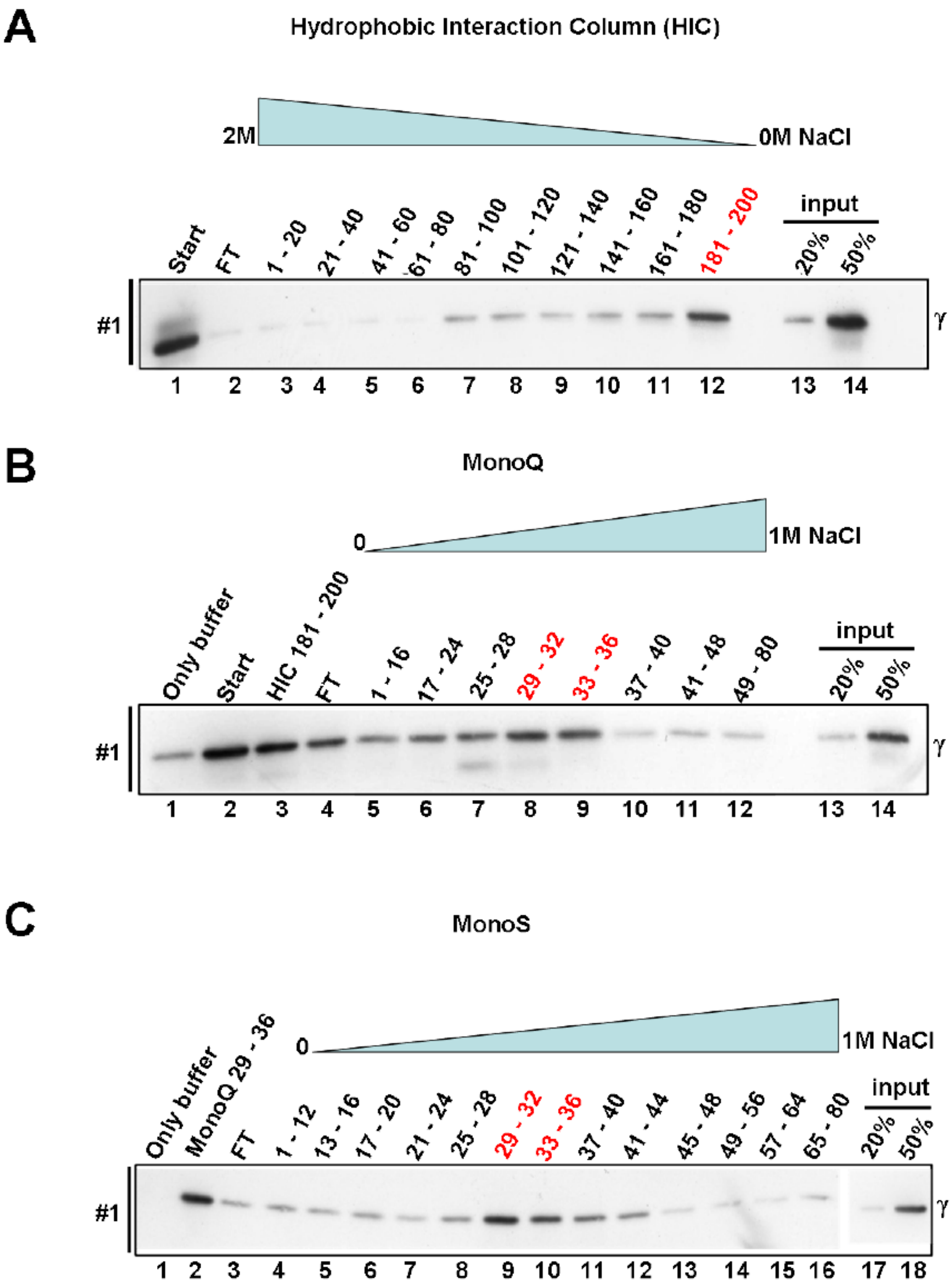

Figure 8. **Purification of the factor:**

(A) The factor-containing fraction of a 30% (sat.) ammonium sulfate precipitation (Start) was separated by hydrophobic interaction chromatography (HIC). Unbound proteins were collected in the flow through (FT) whereas bound proteins were eluted in a linear 200 ml gradient with decreasing NaCl concentrations from 2-0 M NaCl. 132 µg of each fraction was tested for the presence of the factor using the liposome recruitment assay. Lane 13 and 14 represent different amounts of mixed adaptors input. (B) Factor-containing fractions (indicated in red) from the HIC were desalted and loaded on a MonoQ column. Bound proteins were eluted in a linear 80 ml gradient from 0-1 M NaCl and 109 µg of each fraction was subjected to the recruitment assay. As a negative control assay buffer instead of a sample fraction was used (lane 1). (C) Factor-containing MonoQ fractions were desalted and separated on a MonoS column. Proteins were eluted in a linear 80 ml gradient from 0-1 M NaCl. 113 µg of each fraction was tested in the recruitment assay. Lane 17 and 18 represent mixed adaptors input.

With the aim of further purifying the factor, the two factor-containing fractions of the MonoQ experiment were pooled and again desalted on a PD-10 column before they were loaded on a cation exchange column (MonoS) that was preequilibrated in assay buffer. Bound proteins were eluted in a linear 80 ml gradient with increasing salt concentrations from 0-2 M NaCl. Again the buffer of the eluted samples was changed to assay buffer to remove NaCl prior to the recruitment assay. Figure 8C demonstrates that the factor contains also some positively charged residues since it strongly bound to the ion exchanger. The two fractions containing proteins that eluted between 29 ml and 36 ml of the gradient showed the strongest factor activity (lanes 9 and 10).

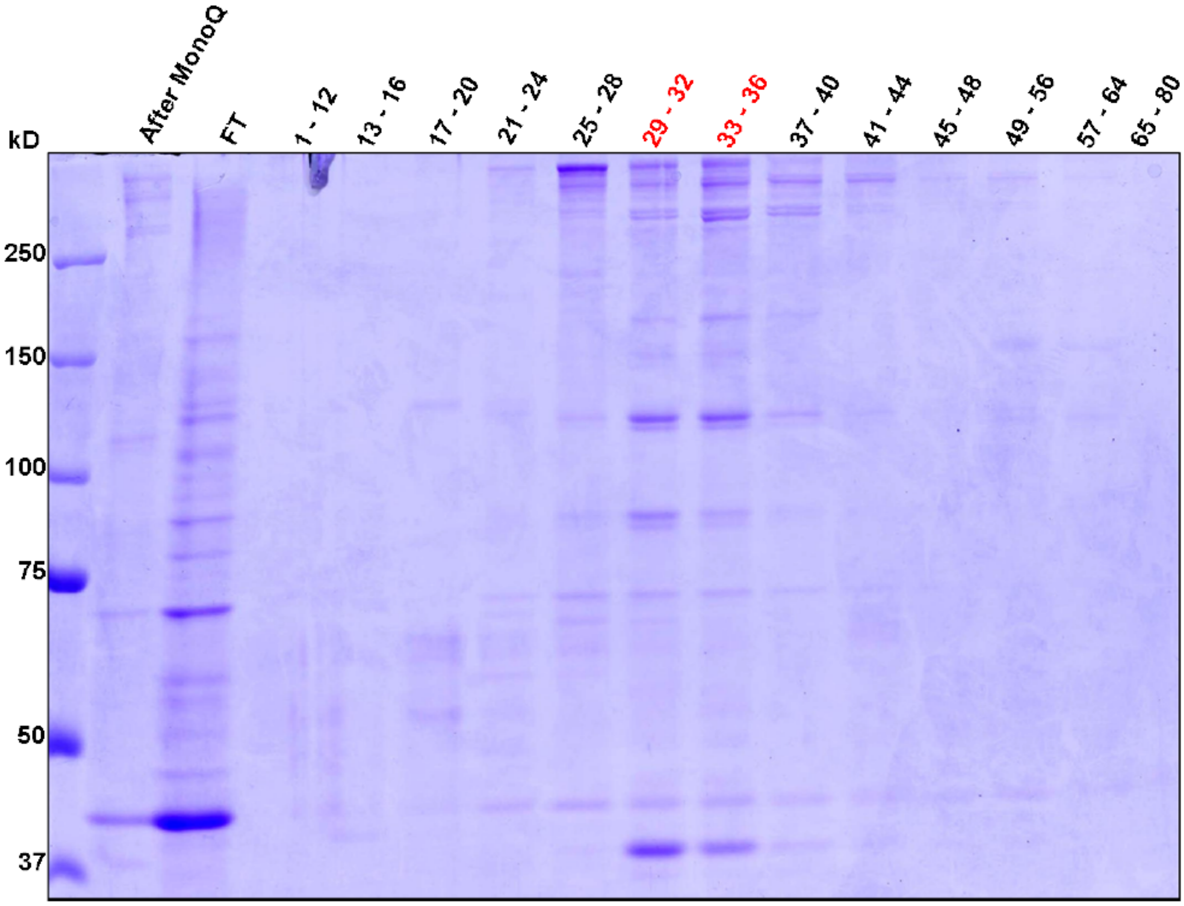

Figure 9. **Coomassie of the MonoS fractions:**
28 µg of each MonoS fraction was analyzed on a Coomassie stained SDS-gel. Factor-containing fractions are indicated in red.

To examine the degree of purification, the MonoS fractions were analyzed by SDS-Page and Coomassie staining. As can be seen in Figure 9, most of the cytosolic proteins were removed from these fractions during the different purification steps. However, to reduce the number of candidates even further, a last separation method was used. Since we have demonstrated that the factor binds to soybean liposomes, a recruitment assay was performed with the MonoS fractions to separate the factor from proteins that were unable to bind to soybean liposomes. Recruited proteins were then analyzed by SDS-page and silver staining and candidates were identified by mass-spectrometry (Figure 10). The strongest bands from the gel were identified as amphiphysin 1, amphiphysin 2, and endophilin A1, three well known accessory factors involved in CCV formation at the plasma membrane. Additionally, α-spectrin, β-spectrin and rho associated protein kinase 2 (ROCK2) were present. However, spectrin could be ruled out for being the cytosolic factor, since it was also present in fraction 37-40 which showed no factor activity. Furthermore, spectrin was one of the contaminating proteins in the AP-1 purification from cytosol which were not able to promote AP-1 recruitment (Figure 2).

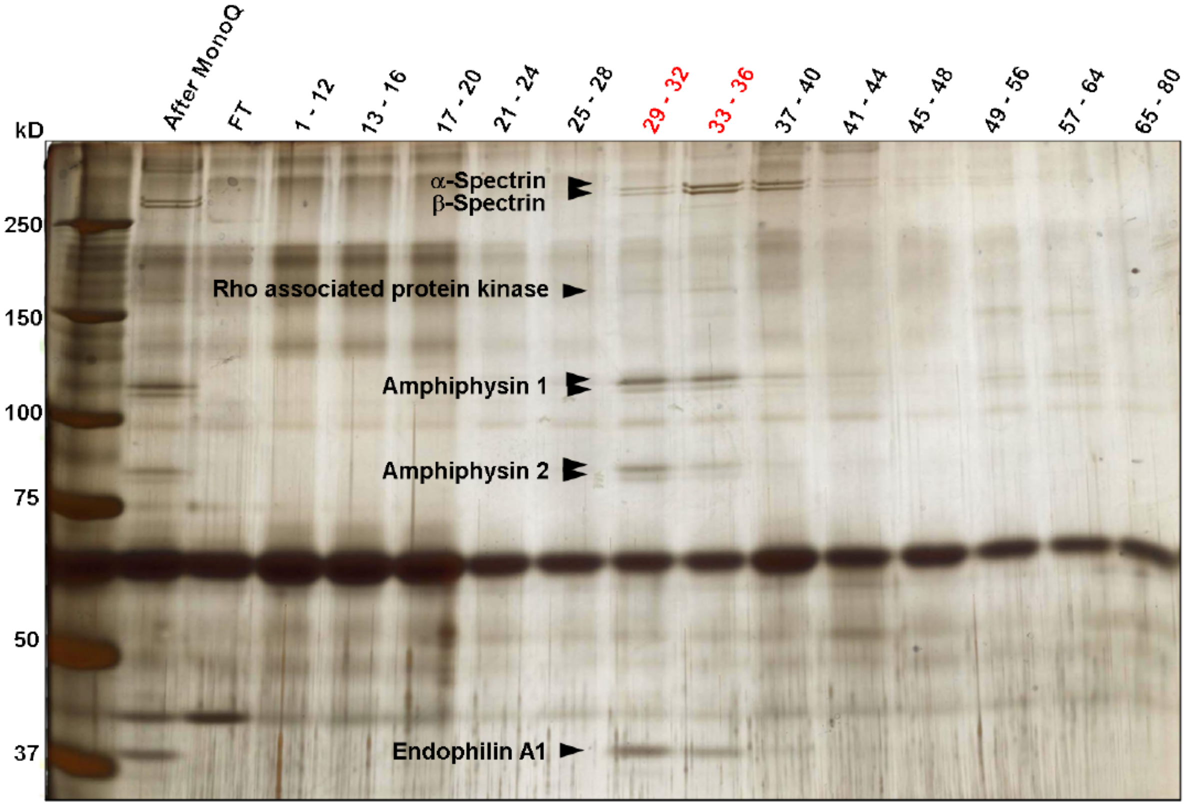

Figure 10. **Silver of the floated MonoS fractions**:

132 µg of the MonoS fractions were subjected to a liposome recruitment assay. After flotation, 80 µl of the top fraction was separated by SDS-gel electrophoresis and stained with silver. Filled arrowheads indicate the position of the proteins that were identified by mass spectrometry and the asterisk marks the position of bovine serum albumin (BSA) which was used as a carrier for TCA precipitation. Factor-containing fractions are indicated in red.

To eliminate some of these remaining factor candidates, an alternative purification method was used. The first two cytosol fractionation steps were the same as before: calf brain cytosol was subjected to a 30% (sat.) ammonium sulfate precipitation before the pellet was dissolved in assay buffer containing 2 M NaCl and separated by HIC. However, as a third purification step the factor-containing HIC fraction was not loaded on an ion exchanger but on a hydroxyapatite column. Bound proteins were eluted in a linear 50 ml gradient with increasing phosphate concentration from 2-500 mM and tested for recruitment activity after the buffer had been changed to assay buffer.

Eluted fractions were not only tested with the flotation assay but equal amounts of the fractions were also analyzed for the presence of amphiphysin 1, amphiphysin 2, and endophilin A1 by immunoblotting. Already during the separation of the proteins by HIC, all three factor candidates were heavily enriched in fraction 161-200, where the main activity of the factor was also measured (Figure 11A, compare lanes 1 and 8). Additionally, several amphiphysin 2 isoforms could be detected (lane 5), which however showed no factor activity. The correlation between factor activity and the presence of the three candidate proteins also remained after the sample had been separated on the hydroxyapatite column (Figure 11B). Some amphiphysin 1 was also present in fractions that did not show AP-1 recruitment (lanes 8-12), but most of the protein was clearly present in the active fractions (lanes 6 and 7).

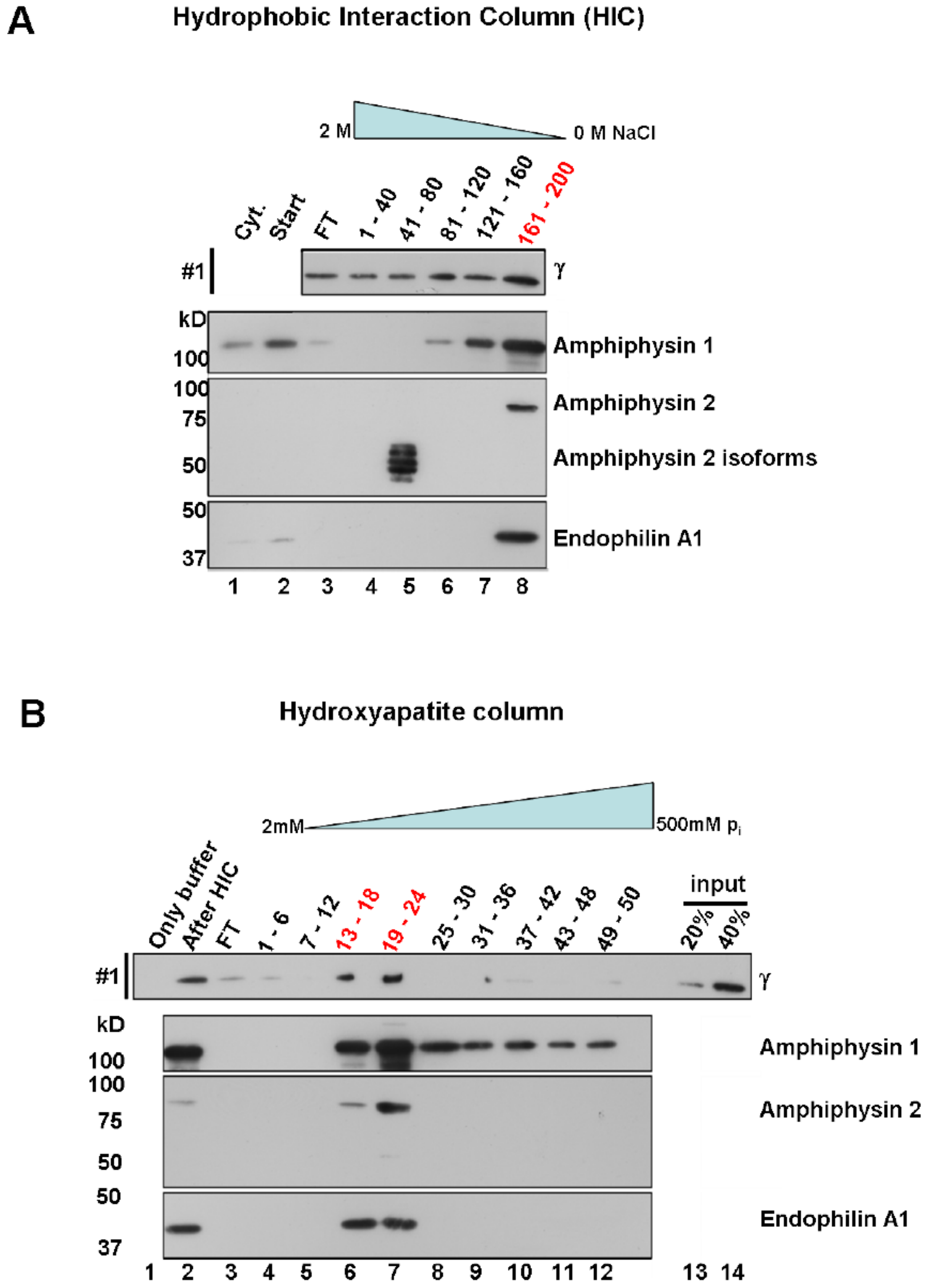

Figure 11. **Alternative purification method:**
(A) A factor-containing sample of a 30% (sat.) ammonium sulfate precipitation of calf brain cytosol (Start) was separated on a HIC. 90 µg of eluted fractions were subjected to a flotation assay whereas another 30 µg were separated by SDS-gel electrophoresis and analyzed by immunoblotting. Unbound material was collected in the flow through (FT). Factor-containing fractions are indicated in red. (B) Factor-containing fractions from the HIC experiment were separated on a hydroxyapatite column. Proteins were eluted in a linear 50 ml gradient from 2mM to 500 mM phosphate. 90 µg of each fraction were used in a recruitment assay, whereas 30 µg were directly analyzed by immunoblotting. As a negative control assay buffer was used instead of a factor-containing fraction (lane 1). Lane 13 and 14 represent different percentages of mixed adaptors input.

To reduce the number of possible candidates in the active fractions, a recruitment assay was performed with the eluted hydroxyapatite samples, to remove proteins that did not bind to the liposomes. Floated proteins were analyzed by SDS-page and silver staining and identified by mass spectrometry (Figure 12). The most prominent bands were again identified as amphiphysin 1 and 2. Unfortunately, endophilin A1 could not be detected on this gel because it runs at ~ 40 kD. However immunoblotting experiments from Figure 11B demonstrated that it is highly enriched in this fraction. Next to these three main candidates, other proteins like clathrin, the two spectrin subunits and microtubule associated protein 2 were detected. However, neither clathrin nor microtubule associated protein 2 were found in the active fractions from the ion exchange columns (Figure 10). Thus in summary these data suggest that, amphiphysin 1, amphiphysin 2 and endophilin A1 are strong candidates for being the cytosolic factor.

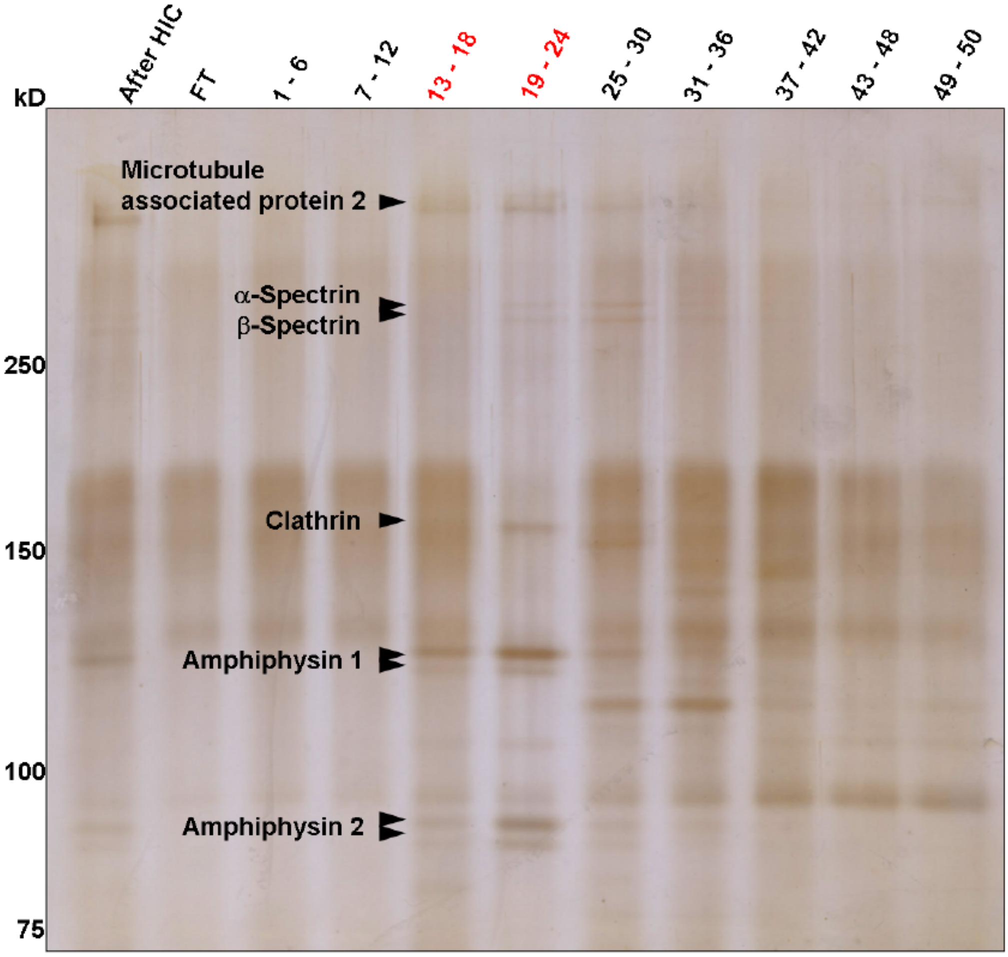

Figure 12: **Analysis of floated hydroxyapatite fractions:**

90 µg of each eluted fraction from the hydroxyapatite column was subjected to a recruitment assay. After the flotation, 80 µl of the top fraction were loaded on a SDS gel and stained with silver. Filled arrowheads mark the proteins identified by mass spectrometry. Factor-containing fractions are indicated in red.

AP-1 is recruited independently of AP-2

The three main candidate proteins identified above are known to be involved in the formation of CCVs at the plasma membrane and two of them, amphiphysin 1 and 2, are known to even bind directly to AP-2. Therefore we were surprised to find these proteins as candidates for a factor that helps to recruit AP-1.

Brain cytosol as well as our adaptor preparation from brain CCVs contains about three times more AP-2 than AP-1. To exclude the possibility that AP-1 was recruited via AP-2, brain cytosol was fractionated as before with a 30% (sat.) ammonium sulfate precipitation, followed by a HIC and MonoQ chromatography. This resulted not only in an enrichment of the factor but also in the removal of cytosolic AP-1 and AP-2 (Figure 13A, lane 4). The factor-enriched MonoQ fraction was then incubated for 30 min at 37°C with soybean liposomes, Arf1-His$_6$, GMP-PNP, and AP-1 that was purified from either CCVs or brain cytosol. After flotation, liposomes with recruited material were collected and analyzed by immunoblotting. As a positive control the experiment was also performed with our standard coat derived AP preparation that in contrast to the purified AP-1 samples still contained AP-2 (compare lanes 1, 2 and 3). As can be seen in Figure 13B, AP-1 was also recruited in the absence of AP-2 (lanes 2 and 5), demonstrating that AP-1 recruitment to soybean liposomes was not an AP-2 dependent artefact.

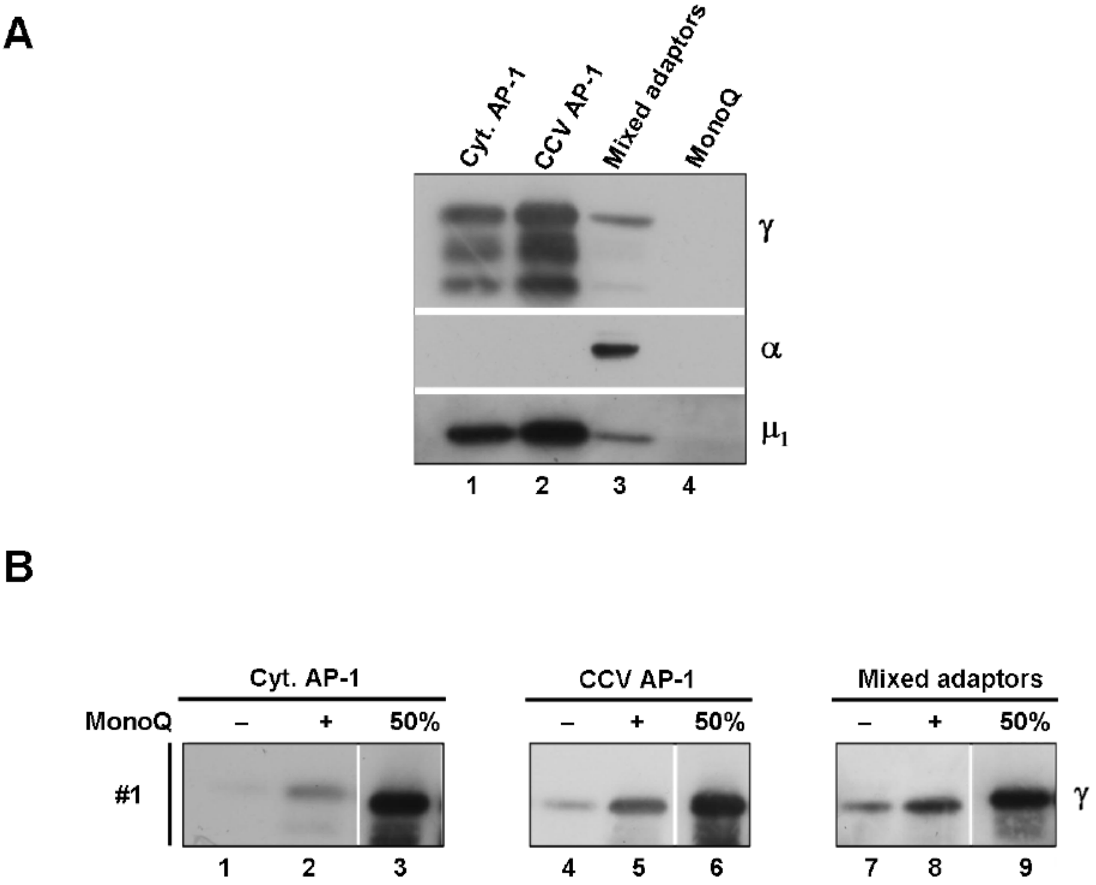

Figure 13. **AP-1 recruits independently of AP-2:**

(A) 3 µg of AP-1 that was either immunopurified from calf brain cytosol (Cyt. AP-1) or hydroxyapatite purified from CCVs (CCV-AP-1), 10 µg of a mixed adaptor preparation and 54 µg of a factor-containing MonoQ fraction that was prepared as described earlier, were analyzed by SDS-PAGE and immunoblotting for the AP-1 subunits (γ, and μ_1) and the AP-2 subunit α. (B) 90 µg of the MonoQ fraction were incubated for 30 min at 37°C with soybean liposomes, Arf1-His_6, GMP-PNP and either cytosol-derived AP-1, CCV-derived AP-1 or mixed adaptors before liposomes were floated in the recruitment assay. After flotation the liposomes were collected and analyzed by immunoblotting (lane 2, 5, and 7). As a negative control, assay buffer was used in the recruitment assay instead of the factor-containing MonoQ fraction (lanes 1, 4 and 7). Lanes 3, 6 and 8 represent 50% of the corresponding AP-1 input.

The β_1- appendage of AP-1 is not needed to interact with the factor

One important feature the cytosolic docking factor should have, is the ability to interact with AP-1. Amphiphysin 1 and 2 interact with AP-2 via its α-appendage domain. To test the involvement of the C-terminal appendage domains of the large γ and β_1 subunits of AP-1 in factor dependent membrane recruitment, a limited trypsin proteolysis of purified AP-1 was performed. As shown in Figure 14A, the appendage domain of β_1 was efficiently removed after limited trypsin proteolysis, whereas the μ_1 subunit and the γ-appendage remained intact. Both, the non-treated AP-1 and the trypsinyzed AP-1 recruited to soybean liposomes in the presence of activated Arf1-His$_6$ and a MonoQ fraction enriched in the cytosolic factor free of AP-1 (Figure 14B). These results demonstrate that the β_1-appendage is not needed to interact with the cytosolic factor, however, it might still interact with AP-1 via its γ-appendage. Unfortunately we could not address this point, since we did not succeed in removing the γ-appendage without digesting μ_1.

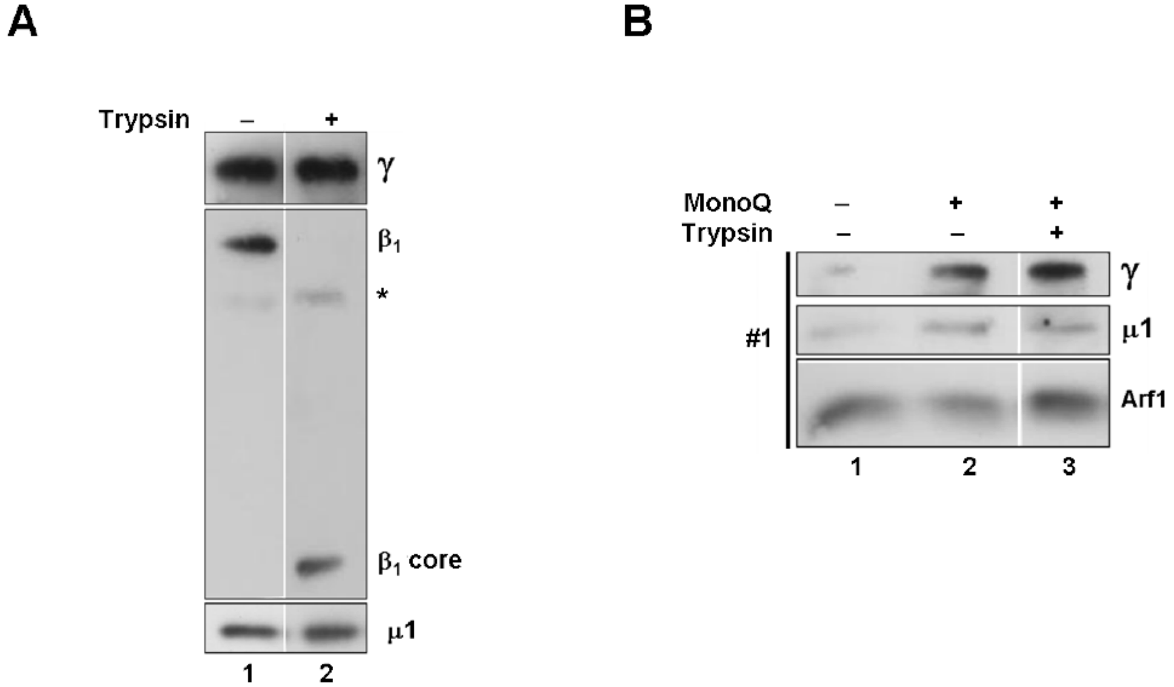

Figure 14: **β_1 is not required to interact with the cytosolic factor:**

(A) Immunopurified cytosolic AP-1 adaptors were subjected to limited proteolysis with trypsin (lane 2) or incubated without protease (lane 1). The products were analyzed by immunoblot analysis using antibodies directed against the hinge segment of γ-adaptin, against the core domain of β_1-adaptin, or against the μ_1 subunit. The band marked with the asterisk resulted from inefficient stripping of the γ-adaptin antibody prior to immunoblotting using the antibody directed against β_1. (B) 2 μg of the treated AP-1 sample were used in a recruitment assay, using a MonoQ fraction as a source of the factor (lane 3). As a positive control 2 μg of untreated AP-1 was tested in the assay (lane 2). Assay buffer that was used instead of the MonoQ fractions served as a negative control (lane 1).

Complex formation of amphiphysin 1, amphiphysin 2 and endophilin A1

By gel filtration the cytosolic factor behaved as a complex apparently larger than AP-1, which has a molecular weight of 260 kD. The three factor candidates have predicted molecular weights of only 76 kD, 65 kD and 40 kD, respectively. Thus, they have to be part of a larger complex. Amphiphysin 1 and 2 have previously been shown to be able to form stable heterodimers via their N-terminal BAR domain, and endophilin A1 is known to form homodimers and to interact with the central insert domains of both amphiphysin isoforms (see introduction).

To test complex formation of the three factor candidates two independent methods depicted in Figure 15 were performed. In a first experiment we performed a sucrose-density gradient centrifugation (Figure 15 A). A factor enriched MonoQ fraction prepared as described above was loaded on top of a linear 10-25% sucrose gradient. After centrifugation at 90’000 x g for 48 h, ten fractions were collected from the top and analyzed by immunoblotting. As a molecular weight standard, we also analyzed AP-1 from mixed adaptors. Amphiphysin 1, amphiphysin 2 and endophilin A1 all co-sedimented and were mainly detected in fractions 4-6 of the gradient, suggesting that they are together in a complex. Surprisingly, AP-1 moved deeper into the gradient and was mainly detected in fractions 7 and 8, suggesting that the complex of the three factor candidates is smaller than AP-1. A possible explanation for the discrepancy between the size of this complex and the size of the factor determined in the gel filtration experiment using full cytosol, is that the shape of the complex affects the hydrodynamic properties and/or the behaviour in gel exclusion abnormally. In this context, it is interesting to note that both amphiphysins show abnormal migration behaviour upon SDS-gel electrophoresis: amphiphysin 1 migrates at 128 kD and amphiphysin 2 at 85 kD. Therefore it is possible that during the gel filtration experiment they also did not migrate according to their predicted sizes. An alternative possibility is that some interaction partners like dynamin or synaptojanin were removed during the purification process.

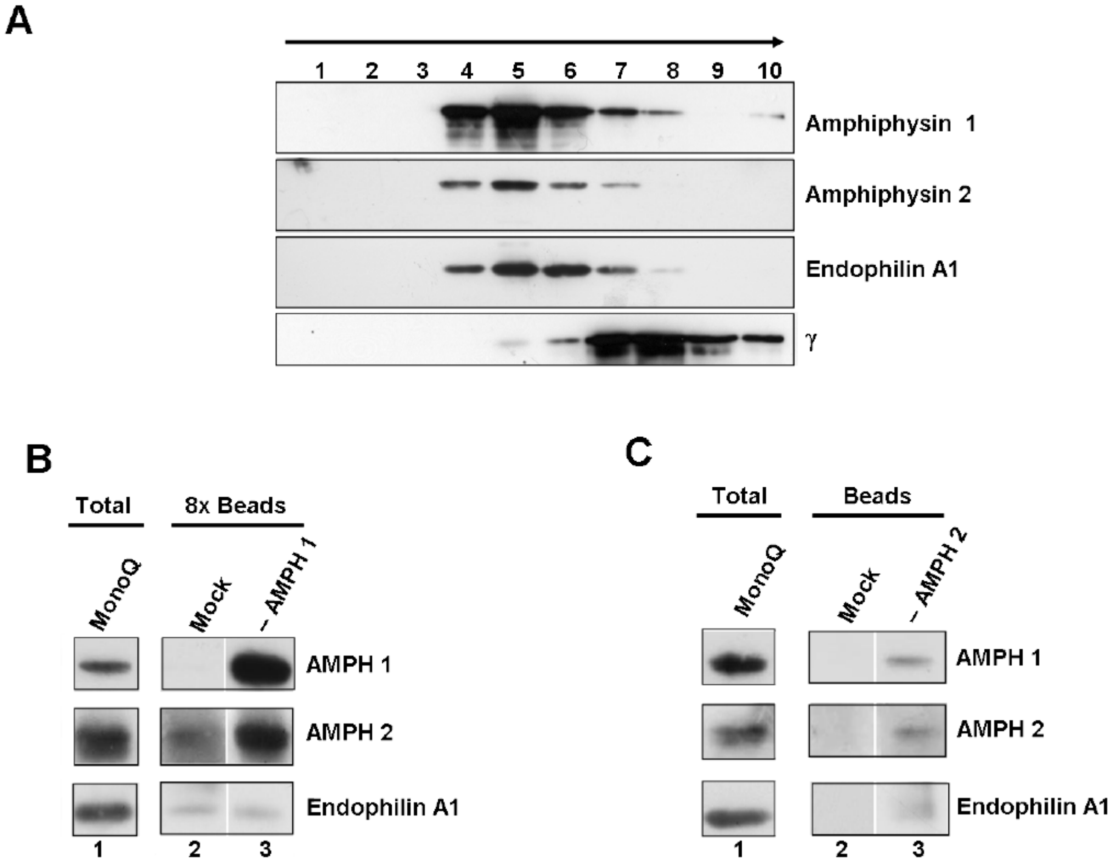

Figure 15. **Complex formation of the three factor candidates:**
(A) A factor-containing MonoQ fraction was centrifuged into a 10-25% sucrose velocity gradient for 48 h at 90’000 x g (horizontal arrow). Ten fractions were collected and analyzed by immunoblotting for amphiphysin 1, amphiphysin 2 and endophilin A1. In order to have a molecular weight standard, the experiment was repeated with a mixed adaptor preparation instead of the MonoQ fraction. The fractions were then analyzed by immunoblotting for γ-adaptin. (B) Co-immunoprecipitation of amphiphysin 1 with amphiphysin 2. 80 µg of a factor-containing MonoQ fraction were immunoprecipitated with an anti-amphiphysin 1 antibody. As a mock empty sepharose A beads were used. Immunoprecipitated pellets (beads) and 10µg of the starting material (total) were analysed by immunoblotting for amphiphysin 1 (AMPH 1), amphiphysin 2 (AMPH2) and endophilin A1. (C) As described in (B) with the difference that an antibody directed against amphiphysin 2 was used in 60 µg of a factor-containing MonoQ fraction. Beads and 50 µg of the untreated sample were analysed by immunoblotting.

To further analyze complex formation between the three proteins, we performed co-immunoprecipitation experiments using a factor enriched MonoQ fraction and antibodies directed against amphiphysin 1 or amphiphysin 2 respectively (Figure 15B and C). Unfortunately, we were not able to perform co-immunoprecipitation experiments with endophilin A1 due to the lack of a suitable antibody (data not shown).

While the antibody directed against amphiphysin 1 efficiently co-immunoprecipitated amphiphysin 2, the antibody directed against amphiphysin 2 was less efficient. However, in both cases, endophilin A1 was not recovered. This rather suggests that endophilin is not stably associated with amphiphysin 1 and 2 or that its association is sensitive to subtle changes in the conditions during immunoprecipitation.

Analytical ultracentrifugation studies provided evidence that endophilin A1 can form high molecular weight oligomers at high concentration (Gallop et al., 2006). Thus it might be that an oligomer consisting of several endophilins moved by chance to the same fraction in the sedimentation gradient as the amphiphysin dimer (Figure 15A). Further studies will be needed to finally demonstrate if endophilin A1 is part of a complex with amphiphysin 1 and 2, or not.

Co-depletion of amphiphysin 1 and 2 reduces AP-1 recruitment

In order to examine whether the removal of the amphiphysins has an effect on AP-1 recruitment, a factor enriched MonoQ fraction was depleted of the amphiphysins, using the antibody directed against amphiphysin 1, before a recruitment assay was performed (Figure 16).

While mock treatment already resulted in some reduction of AP-1 recruitment (lane 3), amphiphysin depletion further diminished recruitment to almost background levels (compare lanes 1 and 4). When we used full cytosol to detect the amphiphysins, only trace amounts of amphiphysin 1 and no amphiphysin 2 could be detected (Figure 11 A, lane 1), suggesting that the antibodies used for immunoblotting were not very sensitive. Thus, the fact that we could not detect any amphiphysin 1 or 2 in the depleted MonoQ fraction does not necessarily mean that there was no amphiphysin left in the sample which could explain why the depleted MonoQ fraction still recruited AP-1 to a minor level. An alternative possibility is that endophilin A1, which is still present in the MonoQ fraction, is responsible for the remaining activity. However we consider it unlikely that endophilin A1 on its own can mediate AP-1 recruitment, since it has (in contrast to the amphiphysins) no AP binding sites. In summary, these findings strongly indicate that a heterodimer of amphiphysin 1 and 2 is necessary to efficiently recruit AP-1 to soybean liposomes in the absence of signal tails.

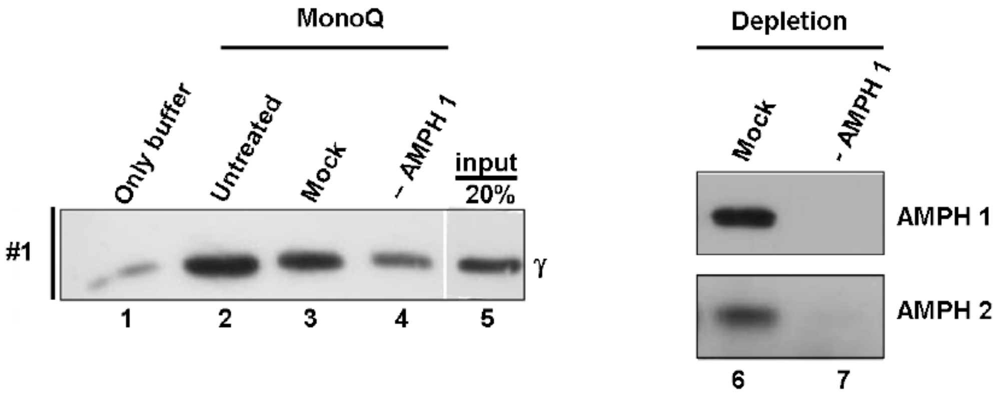

Figure 16. **Amphiphysin 1 and 2 are necessary to recruit AP-1 in the absence of sorting signals:**
A factor-containing MonoQ fraction was immunodepleted using an antibody directed against amphiphysin 1 before 20 µg of the treated fraction were tested in a recruitment assay (lane 4). As a control, 20 µg of untreated or mock treated MonoQ fraction was tested (lanes 2 and 3). As a negative control assay buffer was used instead of a factor-containing fraction (lane 1). Lane 5 represents 20% of coat derived adaptor input. 10 µg of the amphiphysin 1 or mock treated MonoQ fraction was analyzed by immunoblotting (lanes 6 and 7). For the mock experiments empty sepharose A beads were used.

Discussion

Amphiphysin 1, 2 and endophilin A1 mediate AP-1 recruitment to liposomes

The Kornfeld laboratory demonstrated that in addition to activated Arf1, a cytosolic factor is needed to recruit AP-1 to soybean liposomes in the absence of sorting signals (Zhu et al., 1999b). These findings were confirmed by two other reports (Crottet et al., 2002; Meyer et al., 2005), however, the identity of the factor remained elusive. In the present study, we used different purification strategies in order to isolate the factor from calf brain cytosol. During the purification, the activity of the factor was followed with a liposome recruitment assay and the final active fractions were analyzed by mass spectrometry. These fractions contained three major proteins: amphiphysin 1, amphiphysin 2 and endophilin A1. All of them have already been shown to be involved in CCV formation at the plasma membrane. Our finding strongly suggests that amphiphysin 1, 2 and endophilin A1 also participate in the formation of AP-1 containing clathrin coats at the TGN and endosomes.

All three proteins belong to the BAR family (named after the founding members Bin1, Amphiphysin, and Rvs167) and are highly enriched in the brain where they are concentrated in presynaptic nerve terminals. At the N-terminus, they contain an N-BAR domain which consists of a predicted N-terminal amphiphatic α-helix followed by a BAR domain (Figure 17 A). The structures of the BAR domains of amphiphysin 1, 2 and endophilin A1 have been determined by crystallographic analysis (Casal et al., 2006; Peter et al., 2004; Weissenhorn, 2005) and revealed that they consist of three α-helices in a coiled coil arrangement that form a banana shaped dimer (Figure 17 B). The BAR domain of endophilin A1 was shown to mediate homo and heterodimerization with the closely related protein endophilin A2 (Ringstad et al., 2001) and in vitro studies using recombinant BAR domains of amphiphysin 1 and 2 demonstrated that also both amphiphysins can form homo- and heterodimers (Ramjaun et al., 1999; Slepnev et al., 1998). In our active fractions, we found that amphiphysin 2 was completely coimmunodepleted with amphiphysin 1 (Figure 16), suggesting that all of it existed in a heterodimer and no free or homodimeric amphiphysin 2 was present. This is in agreement with studies from di Paolo and colleagues who demonstrated that at least in the brain amphiphysin 2 is only stable in a heterodimer with amphiphysin 1 (Di Paolo et al., 2002).

Analytical ultracentrifugation experiments determined that amphiphysin and endophilin A1 dimers bind with a K_d of 6 μM and 10 μM respectively, suggesting that there is a monomer/dimer equilibrium in vivo, and it was proposed that they only dimerize upon membrane recruitment (Gallop et al., 2006; Peter et al., 2004). However, this is in contrast to our immunoprecipitation experiment where we showed that amphiphysin 1 and 2 dimerize also in the absence of liposomes (Figure 15 B and C). Furthermore, we did not observe any monomeric forms of the factor candidates in the sedimentation assay, using a factor enriched MonoQ fraction (Figure 15 A). Thus monomeric forms might have been purified away.

In addition to being required for dimerization, the BAR domain, in cooperation with the N-terminal α-helix, is also known to mediate strong membrane binding, which in the case of amphiphysin 1 and 2 even resisted high salt washes of 1M KCl or 0.5M Tris-HCl (Lichte et al., 1992; Ramjaun et al., 1999). It was shown that in the absence of the N-terminal α-helices, membrane association of the positively charged concave surface of the BAR dimer with the negatively charged membrane is only mediated via simple electrostatic interactions. Only the presence of the amphiphatic α-helices rendered this interaction salt insensitive (Gallop et al., 2006). Because of the rigid banana shaped BAR domain structure, it is thought that membrane association of the BAR dimer is responsible for its property to curve membranes (Gallop and McMahon, 2005; McMahon and Gallop, 2005; Peter et al., 2004) (Figure 17 C). Furthermore, lipid binding studies revealed preferential binding to small liposomes, suggesting that the BAR domain is not only perfectly designed to influence membrane curvature but also functions as a curvature sensor (Peter et al., 2004).

At the C-terminus, both the amphiphysins and endophilin A1 have an SH3 domain which was shown to interact with the proline rich domains (PRD) of the large GTPase dynamin (100 kD) (David et al., 1996; Leprince et al., 1997; Ringstad et al., 1997) and the lipid phosphatase synaptojanin (145 kD) (de Heuvel et al., 1997; McPherson et al., 1996; Micheva et al., 1997; Ramjaun et al., 1997). We did not find these two proteins in our enriched cytosol fraction as determined by Coomassie and silver staining, which indicates that they did not sufficient interact with amphiphysin 1, 2 and/or endophilin A1 in the cytosol. However, SH3 domain interactions can be very weak (K_d in the order of 1 mM) and therefore it is possible that synaptojanin (which has multiple SH3 binding sites (Cestra et al., 1999)) and dynamin (which forms oligomers) may only interact with amphiphysins and/or endophilin A1 when the latter are concentrated at the highly curved neck of clathrin coated pits and thus present multiple SH3 domains (Olesen et al., 2008).

In contrast to endophilin A1, both amphiphysin 1 and amphiphysin 2 have an additional central insert domain. This region consists of a CLAP domain that was shown to interact with the clathrin heavy chain (Butler et al., 1997; McMahon et al., 1997) and the α-appendage domain of AP-2 through two distinct but partly overlapping sites (Butler et al., 1997; McMahon et al., 1997; Slepnev et al., 2000). In addition it also contains a PRD, which mediates endophilin binding (Micheva et al., 1997). However, even though endophilin A1 is a known interaction partner both of amphiphysin 1 and 2, we obtained conflicting results on whether it is part of a stable complex or not. Endophilin copurified from cytosol after 30% (sat.) ammonium sulfate precipitation, hydrophobic interaction chromatography, two ion exchange chromatographies and a hydroxapaptite chromatography. However, we were unable to coimmunoprecipitate endophilin A1 from a factor-enriched MonoQ fraction using antibodies directed against amphiphysin 1 or 2 (Figure 15 B and C).

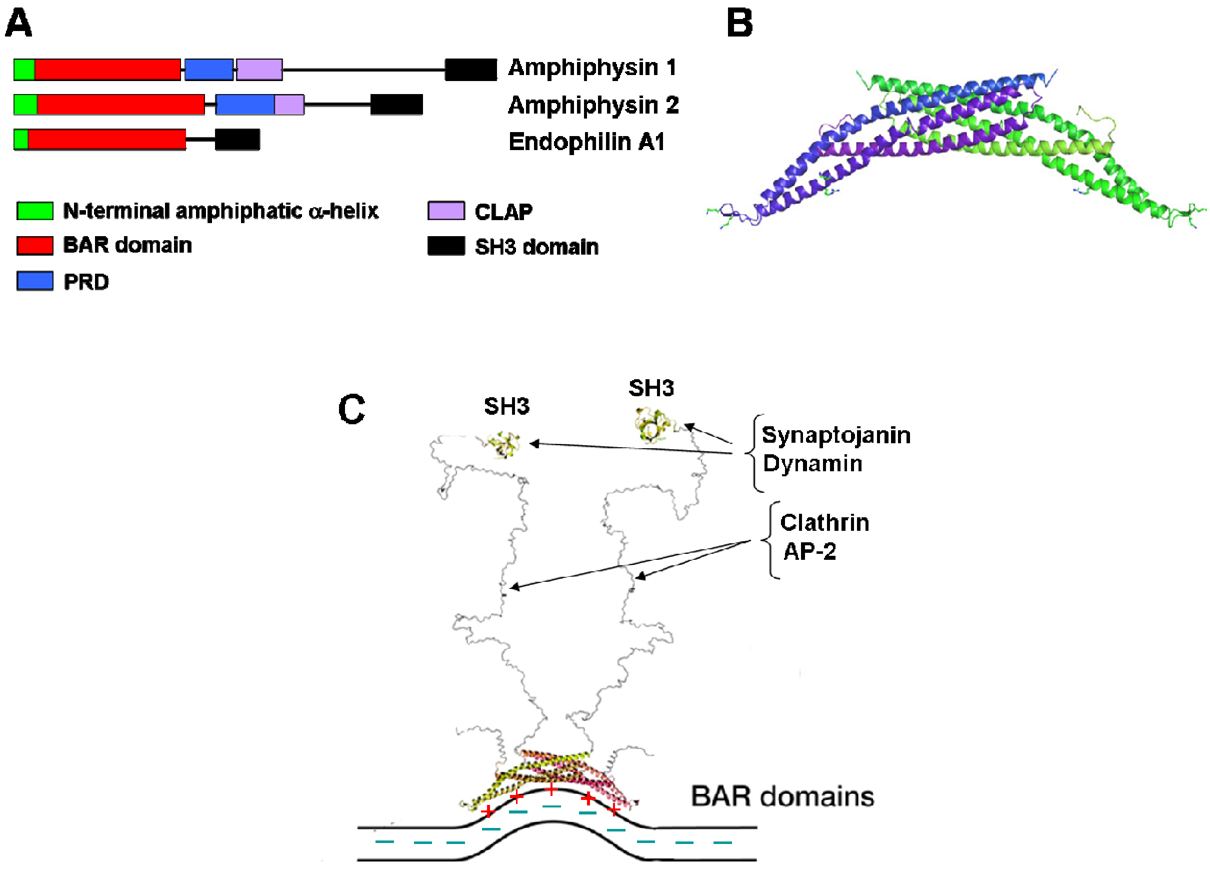

Figure 17. **Structure of the three factor candidates**:
(A) Domain model of amphiphysin 1, 2 and endophilin A1. (B) Crystal structure of Drosophila amphiphysin 1 BAR domain. The two monomers are depicted in green and purple respectively (Peter et al., 2004) (C) Model of amphiphysin 1/2 at the membrane (Owen et al., 2004).

A role for amphiphysins in vesicle formation at the TGN/endosomes?

So far amphiphysin 1, 2 and endophilin A1 have been shown to be involved in CCV formation at the plasma membrane. The exact role of these three proteins is still not clear, but they may contribute to several processes in vesicle formation. The N-BAR domain of amphiphysin 1 and endophilin A1 were shown to tubulate lipid membranes in vitro (Farsad et al., 2001; Takei et al., 1999) and in vivo (Peter et al., 2004). Therefore, the amphiphysins and endophilin A1 may contribute to membrane curvature. In other experiments it was shown that overexpression of amphiphysin 1 or 2 in COS-7 fibroblasts resulted in a potent block of transferrin uptake, which could be rescued by coexpressing dynamin. Interestingly coexpression of both amphiphysins had no effect, suggesting that a heterodimer of amphiphysin 1 and 2 is required to recruit dynamin to the site of vesicle formation (Slepnev et al., 1998; Wigge et al., 1997b). This theory was later supported by the fact that there was a lack of coordination between clathrin coated buds and dynamin coated tubules in the absence of amphiphysins (Takei et al., 1999). Endophilin A1, on the other hand, is thought to recruit synaptojanin, a lipid phosphatase involved in uncoating, since it was shown that the disruption of the endophilin A1 SH3 domain interactions perturbs uncoating of clathrin coated vesicles (Gad et al., 2000).

Our results indicate that amphiphysin 1 and 2 also interact with AP-1. This suggests that the three proteins amphiphysin 1, 2 and endophilin A1 also act in AP-1 mediated clathrin coat formation at the TGN and endosomes where they would perform the same or similar functions. Three reports are in support of this notion. Leprince and colleagues found that amphiphysin 2 colocalized at least in part with early and late endosomal markers (Leprince et al., 2003). In another report it was observed in vitro, that a GST-construct harbouring a short peptide sequence of the distal amphiphysin 2 clathrin binding sequence was able to associate with AP-1 from bovine adrenal cytosol (Drake and Traub, 2001). This interaction was later shown to be direct and to involve the γ-appendage domain of AP-1 (Bai et al., 2004) which is consistent with our finding that the β-appendage domain of AP-1 is not needed to interact with the cytosolic factor (Figure 14). However, the AP-1 binding WXXW motif (which is also found in amphiphysin 1), was also shown to be important for clathrin binding and disruption of the motif resulted in a more than 20 fold impairment of clathrin binding (Slepnev et al., 2000), suggesting that AP-1 and clathrin binding are mutually exclusive.

In our in vitro liposome recruitment system we showed that the depletion of amphiphysin 1 and 2 from a factor enriched MonoQ fraction reduced specific AP-1 recruitment to soybean liposomes to almost background levels (Figure 16). Therefore it seems that the amphiphysins act as a docking factor for AP-1. Neither amphiphysin exhibits a strong lipid specificity that might define the target membrane (Peter et al., 2004). In addition we showed that the recruitment of the cytosolic factor is independent of activated Arf 1 (Figure 4). Therefore, if amphiphysin 1 and 2 act as a docking factor for AP-1 in vivo, the yet unresolved question arises of how these proteins are recruited to the correct organelle. However, it seems that the amphiphysins at least stabilize AP-1 at the membrane.

The model depicted in Figure 18 illustrates the possible functions of amphiphysin 1/2 heterodimers in AP-1 mediated clathrin coated vesicle formation. As a docking factor, amphiphysins together with activated Arf1 recruit AP-1 to the membrane. The curvature generating properties of the amphiphysins may then help in the maturation of the bud into a deeply invaginated pit. The binding of clathrin to AP-1 then is thought to displace the amphiphysins to the bud neck as the coated pit forms, thus targeting dynamin to the correct position. The SH3 domains of amphiphysin 1 and 2 were shown to be able to mediate an intramolecular interaction with the PRD (Farsad et al., 2003; Pineda-Lucena et al., 2005) and it is tempting to suggest that amphiphysins release their SH3 domains only at the neck of forming vesicles to prevent dynamin recruitment at a too early stage. Since it was shown that the interaction of the amphiphysins with synaptojanin is regulated by phosphorylation (Slepnev et al., 1998), the PRD domain may have to be dephosphorylated in order to release the SH3 domain. Amphiphysin 1 was not only shown to recruit dynamin to liposomes, but also to stimulate its GTPase activity in vitro (Yoshida et al., 2004). Interestingly, amphiphysin constructs lacking the SH3 domain (dynamin binding domain) could also stimulate the GTPase activity of dynamin suggesting that the dynamin stimulation is not due to direct amphiphysin 1 interaction with dynamin in solution. Instead it is thought that amphiphysins tubulate membranes with their N-BAR domains which might allow dynamin to co-assemble with amphiphysin around the neck of the forming vesicle (Takei et al., 1999). These rings may represent the dynamin configuration with the maximal GTPase activity which would explain the lack of GTPase stimulation by amphiphysin in solution.

Both amphiphysins are found on highly purified synaptic vesicles (Lichte et al., 1992; Wigge et al., 1997a). However, they are only a minor component of these vesicles and most of the amphiphysins are cytosolic. Thus it is thought that amphiphysins disassociate from the vesicle membrane after fission. Again, phosphorylation might play an important role, since it was shown that the phosphorylation of two residues (Ser-276 and Ser-285) seems to be important to regulate amphiphysin 1 binding to membranes (Liang et al., 2007).

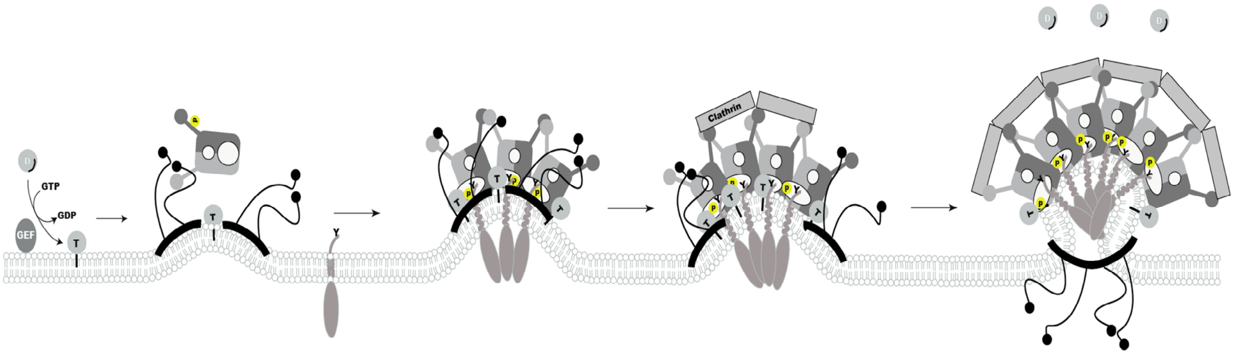

Figure 18. **The potential role of amphiphysin 1/2 heterodimers in AP-1 recruitment**.

Heterodimers consisting of amphiphysin 1 and 2 are thought to recruit AP-1 to the site of vesicle formation were it interacts with PtdIns(4)P and activated Arf1. Cargo binding then results in AP-1 oligomerization. Subsequent clathrin recruitment displaces the amphiphysins to the neck of the forming vesicle where they recruit dynamin. The lower circle in cytosolic AP-1 represents the “inactive” $\mu1$ subunit prior to its phosphorylation. For simplicity ArfGAP, dynamin and other additional accessory proteins involved in AP-1 mediated clathrin coated vesicle formation are not drawn.

Outlook

Future studies will be needed for a more precise characterization of the factor proteins. We have demonstrated that amphiphysin 1 and 2 are required to facilitate AP-1 recruitment in vitro. However, the exact composition of the factor is not entirely established yet. Since amphiphysin 1 can also form stable homodimers it will be very interesting to test, if the complex consists necessarily of an amphiphysin 1/amphiphysin 2 heterodimer, or whether amphiphysin 1 alone is sufficient. Another issue is the participation of endophilin A1. Endophilin A1 is a known binding partner for both amphiphysins and it was one of the three proteins that we isolated from calf brain cytosol. Therefore it may still be a necessary part of the factor complex for AP-1 recruitment, even though endophilin A1 alone is not sufficient (Figure 16). Recruitment assays using different combinations of purified recombinantly expressed amphiphysin 1, 2 and endophilin A1, may reveal the functional composition of the factor complex and prove beyond any doubt that the purified proteins account for AP-1 recruitment.

In order to assess the physiological relevance of our finding, it is necessary to do localization studies to examine if the amphiphysin 1,2 and endophilin A1 colocalize with AP-1.

Furthermore transferrin receptor recycling studies would give insights into the importance of the factor in vivo. Point mutations in the WXXW motif of amphiphysin 2, were shown to abolish in vitro interaction with AP-1 (Bai et al., 2004). Since in the recycling assay clathrin mediated transferrin receptor uptake should not be affected, such constructs that only interfere with AP-1 binding would be ideal. However, since the mutation of this motif also resulted in strongly reduced clathrin binding (Slepnev et al., 2000), one would first have to verify that transferrin receptor uptake is not blocked by this mutation. A cathepsin D secretion assay is an additional test to confirm our findings. Cathepsin D is a cargo of the mannose 6-phosphate receptor. Receptor bound cathepsin D is transported via AP-1 mediated CCVs from the TGN to endosomes from where it is transferred to the lysosome. It is known that impaired AP-1 function results in secretion of cathepsin D. Thus by measuring the amount of secreted cathepsin D in wild type cells and cells without amphiphysins or endophilin A1, we could test the involvement of these three proteins in AP-1 mediated vesicle formation.

According to our current model, both amphiphysins are needed to recruit AP-1 to the correct membrane. However, nothing is known on whether the amphiphysins interact with AP-1 only in the very early steps of vesicle formation until AP-1 is stabilized by cargo, or whether for example they are still part of the AP-1 oligomer which is formed after cargo binding. Therefore it would be interesting to perform a two stage assay, where in a first step AP-1 is allowed to oligomerize in the presence of the LY sorting signals, before in a second step, the solubilized liposomes with the recruited proteins are subjected to a sedimentation assay (Meyer et al., 2005). One could then test, whether amphiphysin 1, 2 and endophilin A1 sediment together with the AP-1 oligomers.

Another interesting subject would involve the phosphorylation of the three proteins. In contrast to endophilin A1 (Micheva et al., 1997), both amphiphysins are heavily phosphorylated in resting neurons (Bauerfeind et al., 1997; Craft et al., 2008; Marks and McMahon, 1998; Slepnev et al., 1998; Wigge et al., 1997a). During nerve terminal depolarization, they are rapidly dephosphorylated by calcineurin (Cousin et al., 2001) which allows them to interact with AP-2, clathrin, endophilin and synaptojanin (Doring et al., 2006; Murakami et al., 2006; Slepnev et al., 1998; Tomizawa et al., 2003). Therefore it would be very interesting to test if AP-1 binding to the amphiphysins is also regulated by phosphorylation for example by preincubating brain cytosol with ATP and phosphatase inhibitors before binding of amphiphysin to a recombinant γ-appendage domain of AP-1.

All three proteins are mainly expressed in the brain. However, the factor activity was also found in bovine adrenal gland and rat liver cytosol (Crottet et al., 2002; Zhu et al., 1999b). Many neuronal proteins, like AP180 or auxilin have ubiquitously expressed homologues. Therefore it will be interesting to determine the composition of an AP-1 recruiting factor in other tissues. Possible candidates would be amphiphysin 1 which was shown to be also expressed in other tissues (e.g. testis, lung, ovary, pituitary, pancreas and adrenal gland) (De Camilli et al., 1993; Wigge et al., 1997a; Wigge et al., 1997b), other members of the endophilin family that show a more general tissue distribution and/or one of the many splice variants of amphiphysin 2 that are ubiquitously expressed. Obviously, only splice variants that retain the central insert domain are likely candidates for this function. Another ubiquitously expressed factor candidate is sorting nexin 18 (SNX18). It belongs to the SNX-BAR family and has a similar, albeit reciprocal, domain structure like the amphiphysins (Cullen, 2008). At the N-terminus it contains an SH3 domain which is followed by a low complexity domain and a C-terminal PX-BAR domain (a BAR domain with an N-terminal phox homology domain). Similar to the N-BAR domain, PX-BAR domains also mediate dimerization and induce curvature upon membrane binding. Lipid specificity is achieved by the phosphoinositide binding phox domain which directs SNX18 to early endosomes. However, SNX18 does not only bind and tubulate membranes but also interacts with dynamin via its SH-3 domain. Furthermore it was shown that it can bind AP-1 with its low complexity domain, thus it is possible that SNX18 has a redundant function for amphiphysins as a docking factor in nonneuronal cells. However, further studies will be needed to reveal if SNX18 recruits AP-1 to endosomes or if AP-1 directs SNX18 to the site of vesicle formation (Haberg et al., 2008).

During the last decade much effort has been put in understanding clathrin coated vesicle formation at the plasma membrane. AP-1 mediated CCV formation, on the other hand, received much less attention. Thus many accessory proteins involved in this process are still unknown. In this study we could shed some light on the accessory proteins involved in AP-1 mediated vesicle formation. Using an in vitro recruitment assay to identify the long searched for determinants that help to recruit AP-1, we found three proteins: amphiphysin 1, amphiphysin 2 and endophilin A1. We showed that at least two of them, amphiphysin 1 and 2, were necessary to recruit AP-1 in the absence of sorting signals. This, together with the fact that all three proteins are known accessory factors in AP-2 mediated CCV formation, strongly suggests that they are also involved in CCV formation at the TGN/endosome.

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